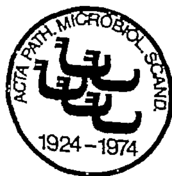
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RADIORESPIROMETRIC STUDIES IN GENUS *NEISSERIA*

2 *The Catabolism of Glutamate and Fumarate*

EIRIK HOLTEN

Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, University of Oslo, Oslo, Norway

Holten, E. Radiorespirometric studies in genus *Neisseria*. 2. The catabolism of glutamate and fumarate. Acta path. microbiol. scand. Sect. B 84 1–8 1976.

The catabolism of glutamate and fumarate was studied by radiorespirometry in selected *Neisseria* species. The tricarboxylic acid cycle is functioning in all species tested, in spite of the known absence of *in vitro* malate dehydrogenase activity in *N. meningitidis*, *N. gonorrhoeae* and *N. cinerea*. The results imply a pyridine nucleotide independent oxidation of malate. The oxidation of glutamate is less complete in the presence of phosphate. In *N. meningitidis*, *N. perflava*, *N. flava*, *N. subflava* and *N. lactamica* the catabolism of fumarate was slow and incomplete in the absence of glutamate.

Key words: Genus *Neisseria*; radiorespirometric studies; glutamate; fumarat; catabolism.

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Radiorespirometric studies have shown that the saccharolytic *Neisseria* species catabolize glucose via the Entner-Doudoroff and the pentose phosphate pathways (5). The presence in these species of a functioning tricarboxylic acid cycle was similarly evident, even in *N. meningitidis*, *N. gonorrhoeae* and *N. cinerea* in spite of their lacking *in vitro* malate dehydrogenase activity (9).

In the present investigation the activity of the tricarboxylic acid cycle has been studied by means of radiorespirometry using specifically ^{14}C-labelled glutamate and fumarate as substrates.

MATERIALS AND METHODS

The strains of *Neisseria* used as well as the radiorespirometric procedure has been described in detail previously (3, 5). In the present study *N. meningitidis* B 8152/66, which lacks glucokinase (4) and aspartase (9), was used in its wild type form.

The experiments were run in KCl/Tris- and phosphate-based minimal media (5). The optimal amount of carrier substrate (L-glutamate and fumarate) varied from strain to strain, and had to be determined in preliminary experiments. The CO_2 trapping solution was changed every 15 minutes. At the end of each experiment an aliquot of the reaction mixture was mixed with scintillation fluid and counted as described (5) to assure that the total recovery of radioactivity was acceptable.

Chemicals. DL-glutamic-1-^{14}C acid, DL-glutamic-2-^{14}C acid, DL-glutamic-3,4-^{14}C acid and DL-glutamic-5-^{14}C acid were purchased from ICN Isotope & Nuclear Division, Irvine, Ca., USA, fumaric-1,4-^{14}C acid and fumaric-2,3-^{14}C acid from The Radiochemical Centre, Amersham, Buckinghamshire, England.

TABLE 1 *Per Cent CO_2 Yield from Specifically Labelled DL-gl tamate at the End of the Experiment*

Species	Medium	µmoles glutamate	Labelled carbon of glutamate: 1	2	3 4	5
N meningitidis						
M 6	KCl/Tris	5	50	38	16	41
	Phosphate	10	48	32	9	38
Ne 15	KCl/Tris	5	51	40	24	45
	Phosphate	5	49	36	15	39
B 8152/66	KCl/Tris	5	52	41	21	44
	Phosphate	5	49	37	18	38
N gonorrhoeae						
1 a	KCl/Tris	3	46	36	2	38
	Phosphate	3	47	29	1	32
21508/70	KCl/Tris	6	50	41	23	46
	Phosphate	6	48	36	18	40
N sicca						
CN	KCl/Tris	10	56	40	18	42
	Phosphate	20	50	29	12	31
N mucosa						
M 4	KCl/Tris	10	53	40	25	45
	Phosphate	20	52	32	16	34
N perflava						
ATCC 10555	KCl/Tris	10	45	36	12	38
	Phosphate	20	49	40	22	43
N flava						
ATCC 14221	KCl/Tris	10	48	38	21	41
	Phosphate	20	48	28	11	28
N subflava						
ATCC11076	KCl/Tris	1	47	35	13	40
	Phosphate	20	49	36	18	39
N lactamica						
ATCC 23970	KCl/Tris	5	50	40	14	41
	Phosphate	12	49	32	1	36
N flavescens						
ATCC 13120	KCl/Tris	15	52	39	23	42
	Phosphate	25	48	26	13	27
N cinerea						
165/61	KCl/Tris	8	50	45	24	47
	Phosphate	15	50	40	13	43
N elongata						
M 2	KCl/Tris	10	50	38	20	44
	Phosphate	20	48	35	11	37
N catarrhalis						
Ne 11	KCl/Tris	6	52	43	25	45
	Phosphate	7	49	40	21	44
N ovis						
199/55	KCl/Tris	5	51	42	25	47
	Phosphate	8	48	41	19	46
N caviae						
ATCC 14659	KCl/Tris	7	47	44	24	47
	Phosphate	10	5	42	26	49

The experiments were terminated when the CO_2 production had ceased, usually after 3 hours.

Fig. 1 Radiorespirometric pattern of the utilization of DL-glutamate by *N. meningitidis* M 6 and B 8152/66 and *N. gonorrhoeae* 21308/70. *KCl* KCl/Tris-based minimal medium. *Phosphate* Phosphate-based minimal medium. DL-glutamate-1-^{14}C ——, DL-glutamate-2-^{14}C: – – –, DL-glutamate-3,4-^{14}C: — — —, DL-glutamate-5-^{14}C ———

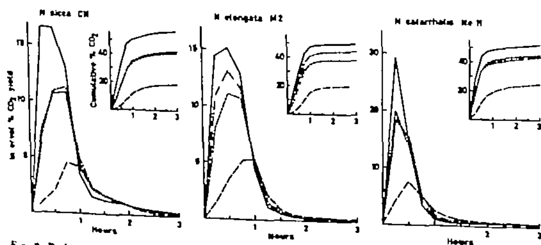


Fig. 2 Radiorespirometric pattern of the utilization of DL-glutamate by *N. sicca* CN, *N. elongata* M 2 and *N. catarrhalis* Ne 11 in KCl/Tris-based minimal Medium. Symbols as in Fig. 1

1*

RESULTS

1 Glutamate

The radiorespirometric patterns of the utilization of specifically ^{14}C-labelled DL-glutamate are shown in Figs. 1 and 2, and the per cent recovery of $^{14}CO_2$ at the end of the experiments in Table 1. The rate and extent of the conversion of carbon atoms of glutamate to respiratory CO_2 follow the order of C1 > C5 ≧ C2 ≫ C3 4 which is consistent with the oxidation of glutamate via the tricarboxylic acid cycle (1 13). Only about 50 per cent of the activity of C1 appeared as CO_2, probably because only L-glutamate is catabolized (13). In all species CO_2 appears rapidly from C1 C2 and C5 less so from C3 4. There is some preferential oxidation of C5 over C2, suggesting a slight asymmetry in the oxidation of the dicarboxylic acids.

The yield of $^{14}CO_2$ was less in phosphate-based medium than in KCl/Tris-based medium, indicating a less complete oxidation. Cells from N. meningitidis M 6 (Fig. 1) and N. *gonorrhoeae* 1 a (not shown) produced CO_2 only very slowly when tested in phosphate-based medium, whereas other strains of these species showed no medium-dependent difference in the rate of CO_2 production. No significant amount of CO was produced from C3 4 by N. *gonorrhoeae* 1 a (Table 1).

Effect of D malate. The influence of D-malate upon the evolution of CO from C3 4 was examined in some strains (Fig. 3). In the "true neisserias" (3) the peak production of CO_2 was delayed (although slightly in N. *flavescens* ATCC 13120) but the total amount of CO_2 formed was not significantly affected. Only a very slight delay was noted in N. *catarrhalis* Ne 11.

2 Fumarate

The radiorespirometric patterns are shown in Figs. 4 and 5 and the per cent recovery of ^{14}CO in Table 2. The differential rate of evolution of CO_2 (C1 4 > C2,3) implies

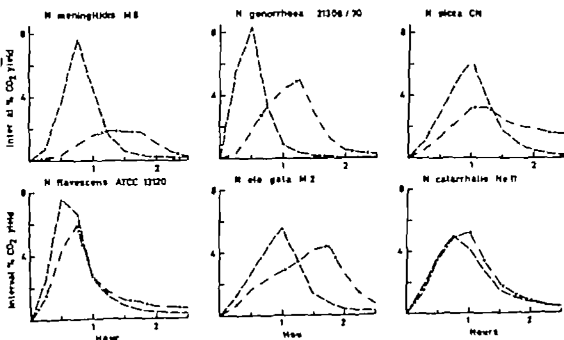


Fig. 3. Radiorespirometric pattern of the utilization of DL-glutamate 3 4 ^{14}C in the absence and presence of D-malate in KCl Tris-based minimal medium. The amount of carrier glutamate is noted in Table 1. DL-glutamate 3 4- ^{14}C ——— DL-glutamate 3 4- ^{14}C plus 10 μmoles D-malate + — —

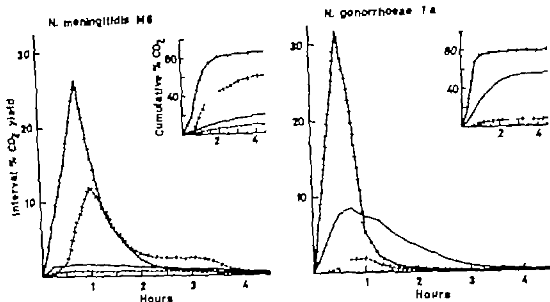


Fig 4 Radiorespirometric pattern of the utilization of fumarate by *N meningitidis* M 6 and *N gonorrhoeae* 1 in KCl/Tris-based minimal medium. Fumarate-1,4-^{14}C ——— fumarate-2,3-^{14}C - - - - fumarate-1,4-^{14}C plus glutamate —+—— fumarate-2,3-^{14}C plus glutamate —+—+—+—

that this substrate is similarly oxidized via the tricarboxylic acid cycle (1)

The production of respiratory CO_2 was slow and incomplete in *N meningitidis* (all strains) *N flava* ATCC 14221 *N subflava* ATCC 11076 and *N lactamica* ATCC 23970 In *N perflava* ATCC 10555 the yield of CO was higher than in these strains, but the radiorespirometric pattern (not shown) resembled that of *N meningitidis* M 6 (Fig 4) and *N subflava* ATCC 11076 (Fig. 5) In the other species, C1 4 *appeared almost* quantitatively as CO_2.

The production of respiratory CO from *fumarate* was greatly stimulated in *N meningitidis* by the addition of glutamate. In *N sicca* CN CO was produced more rapidly in the presence of glutamate, but the total yield was not affected. In *N elongata* M 2 glutamate had no influence on the *rate of* the catabolism of fumarate, but the yield of CO was somewhat reduced (Table 2) probably because of *dilution* of the substrate by unlabelled fumarate derived from glutamate.

DISCUSSION

During the catabolism of fumarate via the tricarboxylic acid cycle, C1 and C4 are liberated as CO in the isocitrate and α keto-glutarate dehydrogenase reactions, with the subsequent appearance of C2 and C3 from the same reactions later As glutamate enters the cell metabolism, it is oxidized to α keto-glutarate (3 8) and CO from C1 will appear from the α-ketoglutarate dehydrogenase reaction. C2 and C5 will then be found as *the carboxyl groups of the* C_4 *dicarboxylic* acids, while C3 and C4 will correspond to the C2 and C3 of these acids (1) The present results, giving the differential rates for the evolution of CO of C1>C5≧C2>C3 4 for glutamate and C1 4>C2,3 for fumarate, are consistent with an operating tricarboxylic acid cycle.

The *finding reported by Tonhazy & Pelczar* (12) that *N gonorrhoeae* can oxidize D-glutamate, is not supported by the present results which indicate that all species tested are able to metabolize L-glutamate only

TABLE 2 *Per Cent CO_2 Yield from Specifically Labelled Fumarate in KCl/Tris-based Medium at the End of the Experiments*

Species	µmoles fumarate	Labelled carbon of fumarate		With glutamate added			
				µmoles unlabelled glutamate	µmoles fumarate	Labelled carbon of fumarate	
		1 4	2,3			1 4	2,3
N. meningitidis							
M 6	5	19	9	5	5	83	60
Ne 15	4	29	19	4	4	88	36
B 8152/66	4	13	3				
N. gonorrhoeae							
1 a	2	56	1	2	2	9	8
21508/70	4	85	32				
N. sicca							
CN	5	89	62	5	5	88	
N. mucosa							
M 4	5	91	64				
N. perflava							
ATCC 10555	5	56	13				
N. flava							
ATCC 14221	5	24	12				
N. subflava							
ATCC 11076	5	16	4				
N. lactamica							
ATCC 23970	5	8	2				
N. flavescens							
ATCC 13120	5	95	72				
[illegible]							
165/61	5	93	41				
N. elongata							
M 2	10	90	64	5	10	86	55
N. catarrhalis							
Ne 11	15	91	66				
N. ovis							
199/55	5	97	68				
N. caviae							
ATCC 14659	15	97	64				

The experiments were terminated when significant CO_2 production had ceased, usually after 3 4 hours.

Phosphate lowers the CO_2 yield from C2, C3 and C3 4 of glutamate whereas the yield from C1 is almost unaffected. Hence the substrate enters the tricarboxylic acid cycle readily but its oxidation is less complete in the presence of phosphate. Also in experiments using specifically ^{14}C-labelled glucose as the substrate the presence of phosphate gave results suggesting a less complete oxidation of acetate via the tricarboxylic acid cycle (5).

It has been demonstrated that several di-

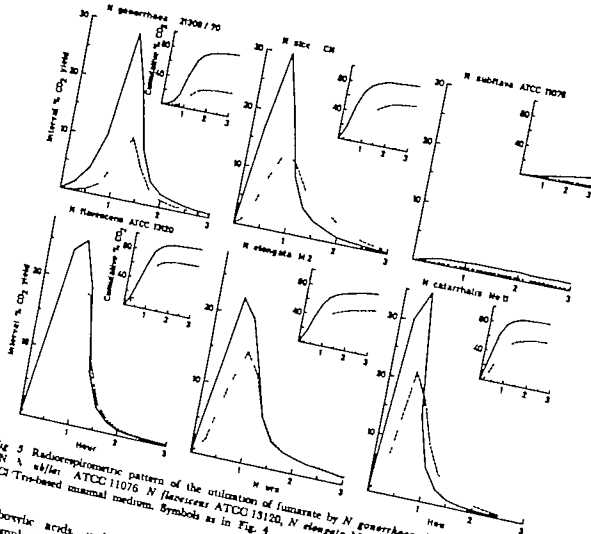


Fig 5 Radiorespirometric pattern of the utilization of fumarate by *N gonorrhoeae* 21308/70, *N sicca* CN *N subflava* ATCC 11076 *N flavescens* ATCC 13120, *N elongata* M 2 and *N catarrhalis* No 11 In KCl Tris-based minimal medium. Symbols as in Fig. 4

carboxylic acids, including fumarate, are incompletely catabolized by *N meningitidis* in the absence of glutamate (14) whereas they are readily oxidized by *N gonorrhoeae* (12) Glutamate has been shown to stimulate the uptake of succinate (14) and citrate (2) in *N meningitidis* and it has been suggested that, in this species, the uptake of succinate and citrate requires energy which is furnished by the metabolism of glutamate (14) The present results conform with this hypothesis.

Both oxaloacetate and acetate are required in order to keep the tricarboxylic acid cycle working. As a consequence both these substances must be synthesized from glutamate or fumarate Pyruvate may be produced from malate by malic enzyme (9 10) acetate thus being produced by the following sequence of reactions fumarate → malate → pyruvate → acetate In *N meningitidis* and *N gonorrhoeae* the crucial point seems to be the synthesis of oxaloacetate. These species do not contain a demonstrable malate dehydrogenase (9 10) hence alternative pathways of oxaloacetate synthesis have been searched for but obviously neither aspartase coupled to aspartate aminotransferase (9) nor phosphoenolpyruvate carboxylase (9 11) seem to be important under the present conditions. Some C compound is formed from pyruvate (6) but it is not known whether this synthesis proceeds via phosphoenolpyruvate The reaction is probably too slow to yield the required amount of oxaloacetate.

Judging from the effect of D malate upon the liberation of CO from the C3 4 of gluta mate malate is probably an intermediate in the production of oxaloacetate when gluta mate is the substrate and recently it has been found that cell free extracts from *N meningitidis* and *N gonorrhoeae* as well as from all other *Neisseria* species tested, do oxidize L-malate to oxaloacetate in the absence of pyridine nucleotides (7)

The activity of the tricarboxylic acid cycle may thus be independent of the malate de hydrogenase. If glucose is used as substrate phosphoenolpyruvate carboxylase is probably important in the production of oxaloacetate. A synthesis of the latter from pyruvate is possible but seems less likely If substrates are catabolized via pathways not including phosphoenolpyruvate as an intermediate oxaloacetate may obviously be produced by pyridine nucleotide independent oxidation of malate.

N gonorrhoeae 1 a is not able to produce CO from C3 4 of glutamate or from C2,3 of fumarate This strain is also unable to liberate CO from C2 and C3 of glucose (5) and pyruvate (6) and it does not oxidize acetate even in the presence of glutamate (6) This can be explained by assuming that this strain has a defective synthesis of citrate Malate and oxaloacetate would then be decarboxylated to give pyruvate and acetate Such decarboxylations would then liberate CO only from C1 and C4 of fumarate while C2 and C3 would appear as acetate Correspondingly C2 and C3 of glutamate would be liberated as CO and C3 4 converted to acetate

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The excellent technical assistance of Miss *Toril Johansen* is gre tly appreciated This investigation ha been supported by a grant from *N g s almenvite skapelige for kn gsråd*

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Acta path. microbiol. scand. Sect. B, 84 9–16, 1976

RADIORESPIROMETRIC STUDIES IN GENUS *NEISSERIA*

3 *The Catabolism of Pyruvate and Acetate*

Eirik Holten

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, University of Oslo, Oslo, Norway

Holten, E. Radiorespirometric studies in genus *Neisseria*. 3. The catabolism of pyruvate and acetate. Acta path. microbiol. scand. Sect B, 84 9–16, 1976.

The catabolism of pyruvate and acetate in selected *Neisseria* species was studied by radiorespirometry Both substrates were oxidized via the tricarboxylic acid cycle. *N elongata* and the "false neisserias" (*N catarrhalis N ovis* and *N caviae*) did oxidize acetate in the absence of other substrates. This can be explained if it is assumed that these species have glyoxylic acid cycle activity In the "true neisserias" other than *N elongata* acetate was oxidized only in the presence of glutamate, indicating that these species do not possess a glyoxylic acid cycle.

Key words Genus *Neisseria* radiorespirometric studies pyruvate acetate catabolism.

Eirik Holten, Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, University of Oslo Oslo, Norway

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The saccharolytic species of *Neisseria* metabolize glucose to pyruvate via the Entner Doudoroff and the pentose phosphate pathways. The pyruvate is readily decarboxylated to acetate which is further oxidized via the tricarboxylic acid cycle (4) The presence of an active tricarboxylic acid cycle has been confirmed by radiorespirometry of specifically ^{14}C-labelled glutamate and fumarate in saccharolytic as well as in non-saccharolytic species of Neisseria (5)

The present paper deals with specific details concerning the catabolism of pyruvate and acetate.

MATERIALS AND METHODS

The strains of *Neisseria* media and radiorespirometric procedures have been described previously (4 5)

Chemicals. The sodium salts of pyruvic 1-^{14}C acid, pyruvic 2-^{14}C acid, pyruvic 3-^{14}C acid, acetic 1-^{14}C acid and acetic 2-^{14}C acid were obtained from The Radiochemical Centre Amersham, Buckinghamshire, England.

RESULTS

1 Pyruvate

The radiorespirometric patterns of the utilization of specifically ^{14}C-labelled pyruvate are shown in Figs. 1 and 2, and the per cent recovery of $^{14}CO_2$ at the end of the experiments in Table 1 The rate and extent of the conversion of carbon atoms of pyruvate to respiratory CO follow the order of C1 > C2 > C3

In KCl/Tris-based medium 80–90 per cent of C1 appeared quickly as CO in all strains tested. This indicates that pyruvate is rapidly and extensively decarboxylated to acetate

TABLE 1 Per Cent CO_2 Yield from Specifically Labelled Pyruvate at the End of the Experiments

Species	Medium	μmoles pyruvate	Labelled carbon of pyruvate			With gl tamate added			
						μmoles unlabelled glutamate	μmoles pyruvate	Labelled carbon of pyruvate	
			1	2	3			2	3
N meningitidis									
M 6	KCl/Tris	40	90	6	5	5	5	51	25
	Phosphate	40	85	32	25	5	5	58	37
Ne 15	KCl/Tris	5	89	43	35	5	5	61	32
	Phosphate	5	84	77	66	5	5	52	37
B 8132/66	KCl/Tris	5	88	6	7	5	5	35	22
	Phosphate	5	89	29	22	5	5	48	37
N gonorrhoeae									
1 a	KCl/Tris	40	57	1	1	10	3	1	1
	Phosphate	20	35	0	0	3	3	1	0
21308/70	KCl/Tris	40	91	9	3	5	5	24	16
	Phosphate	40	83	19	16	5	5	45	27
N sicca									
CN	KCl/Tris	40	90	23	18	10	10	5	41
	Phosphate	40	84	32	28	10	10	48	32
N mucosa									
M 4	KCl/Tris	40	86	38	33	10	10	62	51
	Phosphate	40	83	34	26	10	10	53	34
N perflava									
ATCC 10555	KCl/Tris	40	89	9	8	10	10	10	9
	Phosphate	40	80	24	23	10	10	41	30
flava									
ATCC 14221	KCl/Tris	40	86	16	13	10	10	24	19
	Phosphate	40	77	43	36	10	10	56	45
subflava									
ATCC 11076	KCl/Tris	40	86	22	17	10	10	25	19
	Phosphat	40	78	51	44	10	10	51	41
lactamica									
ATCC 23970	KCl/Tris	30	88	8	7	10	10	21	11
	Phosphate	30	76	11	10	10	10	4	2
N flavescens									
ATCC 13120	KCl/Tris	40	90	20	17	10	10	68	60
	Phosphate	40	83	33	30	10	10	64	49
N cinerea									
163/61	KCl/Tris	30	89	6	4	10	10	34	22
	Phosphate	30	79	10	8	10	10	17	7
N elongata									
M 2	KCl/Tris	30	87	72	53	10	10	51	34
	Phosphat	30	66	62	42	10	10	42	23
N catarrhalis									
Ne 11	KCl/Tris	10	85	74	64	10	10	56	37
	Phosphat	1	31	16	17	5	5	36	30

TABLE 1 *(cont.)*

Species	Medium	µmoles pyruvate	Labelled carbon of pyruvate			With glutamate added			
						µmoles unlabelled glutamate	µmoles pyruvate	Labelled carbon of pyruvate	
			1	2	3			2	3
N. ovis									
199/55	KCl/Tris	10	85	74	64	10	10	53	34
	Phosphate	10	47	35	27	10	10	34	33
N. caviae									
ATCC 14659	KCl/Tris	10	90	78	70				
	Phosphate	10	89	78	64	10	10	70	55

The experiments were terminated when the CO_2 production had ceased usually after 2.5–3 hours.

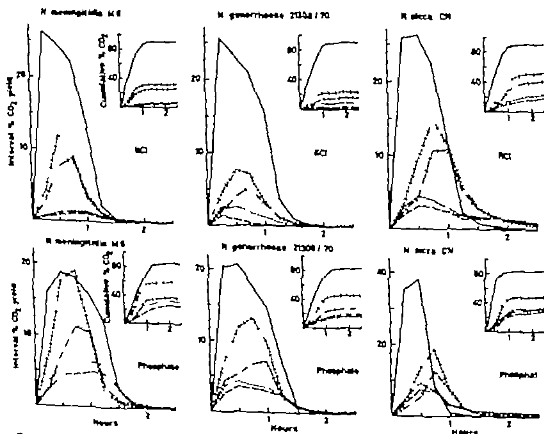


Fig. 1. Radiorespirometric pattern of the utilization of pyruvate by *N. meningitidis* M 6, *N. gonorrhoeae* 21308/70 and *N. sicca* CN. *KCl*: KCl/Tris-based minimal medium. *Phosphate*: Phosphate-based minimal medium. Pyruvate-1-¹⁴C ——— pyruvate-2-¹⁴C – – – ; pyruvate-3-¹⁴C — — — ; pyruvate-2-¹⁴C plus glutamate + + + ; pyruvate-3-¹⁴C plus glutamate —+—+—+—

N flavescens ATCC 13120 · N elongata M 2 · N catarrhalis Ne 11 (KCl)

N flavescens ATCC 13120 · N elongata M 2 · N catarrhalis Ne 11 (Phosphate)

Interval % CO_2 yield · Cumulative % CO_2 · Hour

Fig 2 Radiorespirometric pattern of the utilization of pyruvate by *N flavescens* ATCC 13120 *N elongata* M 2 and *N catarrhalis* Ne 11 Symbols as in Fig 1

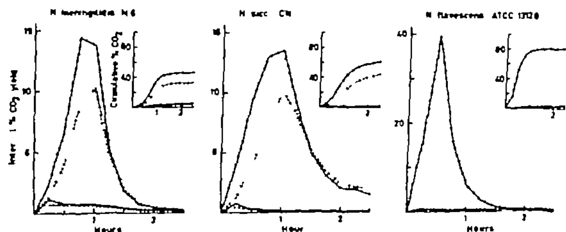


Fig 3 Radiorespirometric pattern of the utilization of acetate by *N meningitidis* M 6 *N sicca* CN and *N flavescens* ATCC 13120 in KCl/Tris-based minimal medium Acetate-1 ^{14}C acetate-2 ^{14}C; – – – acetate-1 ^{14}C plus glutamate acetate-2 ^{14}C plus glutamate

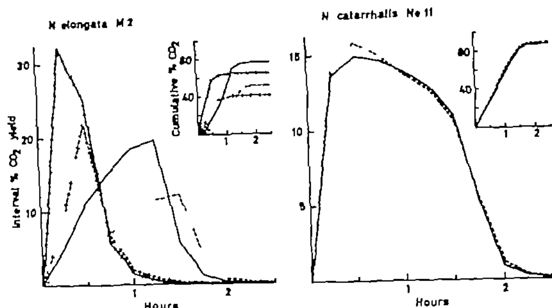


Fig 4 Radiorespirometric pattern of the utilization of acetate by *N elongata* M 2 and *N catarrhalis* Ne 11 in KCl/Tris-based minimal medium. Symbols as in Fig. 3

which is then more slowly catabolized via the tricarboxylic acid cycle (2)

When phosphate-based medium was used, the yield of CO from C1 decreased slightly whereas the production of CO_2 from C2 and C3 was stimulated, particularly in *N meningitidis* and *N gonorrhoeae* In *N elongata* M 2 the evolution of CO from C1 was unaffected while it was somewhat lowered from C2 and C3. In *N catarrhalis* Ne 11 (Fig 2) and *N ovis* 199/55 (not shown) the catabolism of pyruvate was very slow and incomplete in phosphate-based medium, and the height and shape of the curves were largely independent of the concentration of substrate.

The influence of glutamate on the utilization of pyruvate was tested in all strains. The optimal concentration of glutamate had been determined previously (5) In the present experiments equimolar amounts of pyruvate and glutamate was found to give maximal yield of CO (Table 1) In almost all strains of the true neisserias" tested, with the exception of *N elongata* M 2, the yield of CO from C2 and C3 was increased in the presence of glutamate. The production of CO from C1 was not affected. In *N elongata* M 2 the presence of glutamate decreased the recovery of $^{14}CO_2$.

In the "false neisserias" (*N catarrhalis* *N ovis* and *N caviae*) the effect of glutamate resembled that found in *N elongata* M 2 when KCl/Tris-based medium was used, whereas in the phosphate-based medium the catabolism of pyruvate was stimulated by glutamate in *N catarrhalis* Ne 11 and *N ovis* 199/55 In *N caviae* ATCC 14659 using phosphate-based medium, the amount of respiratory CO_2 from pyruvate decreased in the presence of glutamate.

2 Acetate

The radiorespirometric patterns are shown in Figs. 3 and 4 and the per cent recovery of $^{14}CO_2$ in Table 2. In all species of the "true neisserias" tested, with the exception of *N elongata* M 2 only negligible amounts of CO were produced. The presence of glutamate greatly enhanced the production of CO_2, and the differential rate of CO_2 evolution was C1>C2, in accordance with an oxidation via the tricarboxylic acid cycle (2)

In *N elongata* M 2 and the "false ...

TABLE 2 *Per Cent CO_2 Yield from Specifically Labelled Acetate in KCl/Tris-based Medium at the End of the Experiments*

Species	µmoles acetate	Labelled carbon of acetate		With glutamate added			
				µmoles unlabelled glutamate	µmoles acetate	Labelled carbon of acetate	
		1	2			1	2
N meningitidis							
M 6	5	5	4	5	5	46	33
Ne 15	5	10	8	5	5	18	11
B 8152/66	5	2	2	5	5	23	
N gonorrhoeae							
1 a	5	0	0	5	5	7	2
21508/70	5	1	0	5	5	67	
N sicca							
CN	5	2	1	10	5	62	45
N mucosa							
M 4	5	1	0	5	5	70	
N perflava							
ATCC 10555	5	3	1	5	5	21	
N flava							
ATCC 14221	5	2	1				
N subflava							
ATCC 11076	5	1	1	5	5	20	
N lactamica							
ATCC 23970	5	1	0	5	5	9	
N flavescens							
ATCC 13120	5	6	7	5	5	82	
N cinerea							
165/61	5	1	0	4	4	72	
N elongata							
M 2	5	77	52	5	5	66	42
N catarrhalis							
N 11	5	88	89				
N ovis							
199 55	5	88	74	5	5	75	
N caviae							
ATCC 14659	5	91	77	5	5	78	

The experiments were terminated when the CO_2 production had ceased usually after 2.5–3 hours.

seriae" CO_2 was readily evolved with acetate as the sole substrate. Glutamate made the peak production of CO_2 in *N elongata* M 2 appear earlier but the total yield of CO_2 in this strain was less in the presence of glutamate. In the "false neisseriae" the only effect of glutamate was that it decreased the yield of CO_2 (Table 2 not shown in figures).

DISCUSSION

When acetate is oxidized by the tricarboxylic acid cycle C1 will appear as CO_2 first followed by C2. Pyruvate will initially be decarboxylated to acetate with the liberation of C1 as CO_2. The carboxyl and methyl groups of the resulting acetate will then be derived from the carbonyl and methyl groups of pyru-

vate respectively (2). The present results, with differential rates of CO_2 evolution of C1>C2>C3 for pyruvate and C1>C2 for acetate is consistent with the oxidation of these substrates via the tricarboxylic acid cycle.

The activity of the tricarboxylic acid cycle is dependent upon a source of oxaloacetate. In all strains of *Neisseria* tested, the cycle operates with pyruvate as the sole carbon source indicating a synthesis of C_4 compounds from this substrate.

In order to obtain a synthesis of C_4 compounds from acetate, the glyoxylic acid cycle must be present: the production of CO_2 from acetate requires the concurrent operation of the tricarboxylic and the glyoxylic acid cycles (13). When *N. elongata* M 2 and the "false neisserias" are confronted with acetate as the sole carbon source, CO_2 is readily liberated indicating the presence of the glyoxylic acid cycle in these species. The extensive catabolism of C2 and C3 of pyruvate may also confirm the existence of this pathway.

When the "true neisserias" with the exception of *N. elongata* M 2, are presented with acetate only insignificant amounts of CO_2 are liberated. This production of CO_2 can be ascribed to endogenous oxaloacetate present. The results indicate that these strains cannot utilize acetate for synthesis of C_4 compounds, evidently because of the lack of a functioning glyoxylic acid cycle.

Randles (11) found that *N. catarrhalis* was able to oxidize acetate, and *N. catarrhalis*, *N. ovis* and *N. sicca* have been found to grow with acetate as the sole source of carbon (1). In contrast it has been shown that *N. gonorrhoeae* will not oxidize acetate (14) and that *N. meningitidis* cannot utilize acetate as source of energy (12). These findings support the present assumption that the glyoxylic acid cycle is present in the "false neisserias" but is lacking in the "true neisserias" (other than *N. elongata*). *Morse et al.* (10) showed *N. gonorrhoeae* to catabolize acetate. However these investigators used a medium containing peptone and glutamate, substances from which oxaloacetate can be synthesized.

The inability of the "true neisserias" other than *N. elongata* to synthesize C_4 compounds from acetate implies that oxaloacetate is synthesized from pyruvate probably by CO_2 fixation. In *N. meningitidis* CO_2 fixation has been shown to occur by the phosphoenolpyruvate carboxylase (9). Also in the other *Neisseria* species this enzyme is active (8). The phosphoenolpyruvate carboxylase is inhibited by phosphate (9). In the present system, the synthesis of C_4 compounds appears to be stimulated by phosphate since the CO_2 yield from C2 and C3 of pyruvate is increased in phosphate-based medium. This cannot be due to changes in the tricarboxylic cycle activity as the latter decreases in the presence of phosphate (5). It therefore seems unlikely that the synthesis of C_4 compounds from pyruvate should proceed via phosphoenolpyruvate. The synthesis of phosphoenolpyruvate from pyruvate is also considered to be too slow to form phosphoenolpyruvate at the required rate (9).

Because of the rapid and extensive decarboxylation of pyruvate, only a fraction of this substrate will be available for the synthesis of C_4 compounds. This may be the explanation of the relatively low yield of CO_2 from C2 and C3 in the species lacking the glyoxylic acid cycle. Glutamate stimulates the production of CO_2 from C2 and C3 of pyruvate, presumably by providing oxaloacetate via the NAD-dependent glutamate dehydrogenase (3, 7) and the enzymes of the tricarboxylic acid cycle, including the pyridine nucleotide independent malate dehydrogenase (6).

The slow and incomplete catabolism of pyruvate in *N. catarrhalis* Ne 11 and *N. ovis* 199/55 when using phosphate-based medium, may either be due to permeability problems or to an inhibited decarboxylation of pyruvate. The present results do not provide evidence to choose the correct explanation.

The production of CO_2 from C2 and C3 of pyruvate is much delayed in *N. elongata* M 2, compared to that from C1. The C2 and C3 curves resemble those obtained from C1 and C2 of acetate. The activity of the glyoxy-

lic acid cycle thus appears to be dependent on induction in this strain. By adding glutamate oxaloacetate is produced rapidly allowing a swift evolution of CO_2, and the need for synthesis of C compounds from acetate diminishes. The total yield of CO_2 is reduced however probably because of a dilution of radioactive substrate by inactive metabolites derived from glutamate.

Using cells of *N. gonorrhoeae* 1 a no CO_2 appears from the C2 and C3 of pyruvate *nor from acetate. Even in the presence of* glutamate only negligible amounts of CO_2 are produced from acetate. As pointed out previously (5) this may be the effect of an inability of this strain to catabolize acetate possibly because of a greatly impaired synthesis of citrate

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The excellent technical assistance of Miss *Terli Johansen* is greatly appreciated. This investigation has been supported by a grant from *Norges almenvitenskapelige forskningsråd*

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Acta path. microbiol. scand. Sect. B, 84: 17–21, 1976

PYRIDINE NUCLEOTIDE INDEPENDENT OXIDATION OF L-MALATE IN GENUS *NEISSERIA*

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Holten, E. Pyridine nucleotide independent oxidation of L-malate in genus *Neisseria*. Acta path. microbiol. scand. Sect. B, 84: 17–21, 1976.

In cell free extract from *Neisseria meningitidis* an enzyme has been found which catalyses the oxidation of L-malate to oxaloacetate in the absence of pyridine nucleotides, using ferricyanide as electron acceptor. The enzyme was found to be particle-bound, as determined by sucrose gradient centrifugation. Activity corresponding to this enzyme was demonstrated in extracts from all strains tested of selected *Neisseria* species. In contrast to the large differences in NAD-linked malate dehydrogenase activity among the species, the interspecies variation of the pyridine nucleotide independent oxidation of malate was not sufficiently distinct to be useful for classification purposes.

Key words: Genus *Neisseria*; L-malate oxidation; pyridine nucleotide independent.

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Saccharolytic *Neisseria* species catabolize glucose via the Entner-Doudoroff and the pentose phosphate pathways (4). Acetate arising from the decarboxylation of pyruvate is oxidized by the tricarboxylic acid cycle (6) which is shown to be active in saccharolytic as well as in non-saccharolytic species of *Neisseria* (5). Because of the failure to detect any NAD-linked oxidation of malate in extracts from *N. meningitidis*, *N. gonorrhoeae* and *N. cinerea* (8, 13), alternative pathways of oxaloacetate synthesis have been suggested (8, 14). However, by radiorespirometry of specifically ¹⁴C-labelled glutamate, evidence accumulated which strongly indicated a direct oxidation of malate to oxaloacetate (5). A membrane-bound, pyridine nucleotide independent, malate dehydrogenase has been found in many strictly aerobic bacteria (1, 10, 11, 15). In the present report, evidence for a similar enzyme in species of the genus *Neisseria* is presented. Its activity has been studied in the different species in order to examine any correlation to the activity of the NAD-linked malate dehydrogenase (8), and also to collect data for classification purposes.

MATERIALS AND METHODS

The *Neisseria* strains and the extraction procedure have been used in previous experiments (2, 7). Only cells grown on blood agar were used.

Enzyme assays. Pyridine nucleotide independent malate dehydrogenase was assayed according to Benziman (1) in a mixture containing KCl 250 µmoles, KCN 25 µmoles, potassium L-malate 25 µmoles, $K_3F(CN)_6$ 1.5 µmoles, extract 0.05–0.2 ml containing 15–30 mg protein per ml, and Tris/HCl

lic acid cycle thus appears to be dependent on induction in this strain By adding glutamate, oxaloacetate is produced rapidly allowing a swift evolution of CO_2, and the need for synthesis of C compounds from acetate diminishes. The total yield of CO_2 is reduced, however probably because of a dilution of radioactive substrate by inactive metabolites derived from glutamate.

Using cells of *N gonorrhoeae* 1 a no CO_2 appears from the C2 and C3 of pyruvate nor from acetate. Even in the presence of glutamate only negligible amounts of CO_2 are produced from acetate As pointed out previously (5) this may be the effect of an inability of this strain to catabolize acetate, possibly because of a greatly impaired synthesis of citrate.

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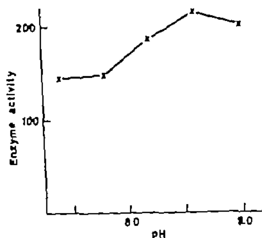


Fig 2 pH optimum for the activity of the pyridine nucleotide independent malate dehydrogenase from *N meningitidis* M 6. Enzyme activity is expressed as nmoles L-malate converted per minute per mg protein.

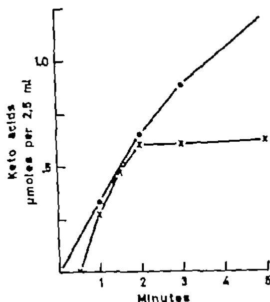


Fig 3 Formation of oxaloacetate and pyruvate from L-malate by extract from *N meningitidis* M 6. The reaction mixture is described under *Materials and Methods*. Aliquots were removed at the times indicated for the analysis of keto acids. Ordinate μmoles keto acid formed per 2.5 ml reaction mixture. Oxaloacetate ×——× pyruvate ○——○

The enzymic activity was unstable. The extract lost 80 per cent of its activity when stored overnight at 4 C. Stored frozen at −20 C about 20 per cent of its activity was lost after 2 weeks.

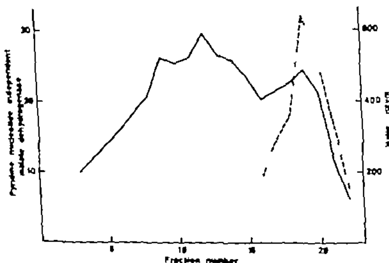


Fig 4 Sedimentation pattern of the pyridine nucleotide independent malate dehydrogenase and malic enzyme in extract from *N meningitidis* M 6 after ultracentrifugation in a linear 5–20 per cent sucrose gradient. 22 fractions were obtained, the top fractions are to the right. Enzyme activities are expressed nmoles L-malate converted per minute per fraction. Pyridine nucleotide independent malate dehydrogenase ——— malic enzyme - - - - -

Two peaks of pyridine nucleotide independent malate dehydrogenase activity were found by sucrose gradient centrifugation of extract from *N meningitidis* M 6 a narrow one, coincident with the peak of the NADP linked malic enzyme in fraction 19 and a broad peak with maximal activity in fraction 12 (Fig 4) The broad peak implies that malate oxidizing activity resides in particles heavier than the soluble molecules of malic enzyme, and that the size of these particles varies. It has been shown that the aerobic bacteria *Moraxella lwoffi Pseudomonas fluorescens Acetobacter aceti Micrococcus lysodeikticus* and some strains of *Bacillus* (9) *Pseudomonas ovalis* (15) *Acetobacter xylinum* (1) and *Azotobacter vinelandii* (11) contain a membrane-bound pyridine nucleotide independent malate dehydrogenase, while facultative microbes (*Klebsiella aerogenes Escherichia coli* and *Proteus vulgaris* (9)) do not have this enzyme. The enzyme from *M lwoffi* (10) and *A vinelandii* (11) seems to be associated with the electron transport chain. It remains to be established whether this is the case also in *Neisseria*.

Some malate oxidizing activity also resides in the soluble fraction, together with the malic enzyme. Owing to a strong lipolytic activity of the extracts from *N meningitidis* membrane particles seem to be unstable (*Jyssum* personal communication) thus explaining why part of the pyridine nucleotide independent malate dehydrogenase seems to be solubilized. In the cases of *N flava* and *N catarrhalis* pyridine nucleotide independent malate dehydrogenase was not demonstrable in the particulate fraction until the supernatant had been added to the system (12) possibly because of a similar lipolytic activity

No attempts were made to determine the nature of the electron acceptor In other systems, the pyridine nucleotide independent malate dehydrogenase is flavoprotein-linked (1 11 15)

All the "true neisserias" (2) including *N meningitidis N gonorrhoeae* and *N cinerea* contain a substance which reacts with antibody to NAD-linked malate dehydrogenase from *N perflava* in double diffusion and complement fixation tests (3) The function of this substance is not known, neither has it been determined whether it is responsible for the oxidation of malate described in this paper However in *M lwoffi* it was shown that the pyridine nucleotide independent and the NAD-linked malate dehydrogenases were two different proteins (10)

TABLE 1 *Activity of the Pyridine Nucleotide Independent Malate Dehydrogenase i Neisseria*

Species	Strain	Enzyme activity
N meningitidis	M 1	191
	M 3	147
	M 6	290
	B 8152/66	203
	Ne 15	207
	ATCC 13113	1 7
	P 5	141
	P 17	3_3
	P 19	233
	P 22	263
	1535	236
	1875	207
N gonorrhoeae	1 a	27
	2 c	124
	44341	241
	21306/70	157
	21319/70	?08
	21332/70	246
N sicca	CN	141
	6	131
	6021	127
N mucosa	M 1	88
	M 4	489
	M 9	296
N perflava	ATCC 10555	53
	A 2	26
	191	39
N flava	ATCC 14221	222
	B	91
	X 4	93
N subflava	ATCC 11076	?95
	ATCC 19243	152
	115	59

TABLE 1 (continued)

Species	Strain	Enzyme activity
N lactamica	ATCC 23970	141
	1579	170
	161 Sc	250
N flavescens	ATCC 13115	351
	ATCC 13117	273
	ATCC 13120	407
N cinerea	165/61	404
	137/6?	29
	159/62	22
N longata	M 2	54
	7823/71	39
	8554/71	45
N catarrhalis	ATCC 8176	47
	Ne 11	62
	13016/62	50
N ovis	199/55	21
	37/39	26
	317/60	34
N caviae	ATCC 14659	111
	NCTC 10293	68

Enzyme activity is expressed as nmoles L-malate oxidized per minute per mg protein.

All *Neisseria* strains tested were able to reduce ferricyanide in the presence of L-malate (Table 1). Relatively low activity was noted in the "false neisserias" (*N catarrhalis*, *N ovis* and *N caviae*), *N elongata*, *N perflava*, and some strains of *N flava* and *N cinerea*. The relatively small variation in the activity of the pyridine nucleotide independent malate dehydrogenase may indicate a similar function in all *Neisseria* species. It sharply contrasts, and is not correlated to, the large interspecies differences in malate dehydrogenase activity (8). As regards the pyridine nucleotide independent malate dehydrogenase the inter- and intraspecies variation found is not sufficiently distinct to be useful in classification.

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OCCURRENCE OF REO-LIKE VIRUSES IN YOUNG CHILDREN WITH ACUTE GASTROENTERITIS

Diagnoses Established by Electron Microscopy and Complement Fixation Using the Reo-Like Calf Virus as Antigen

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Tufvesson, B. & Johnsson, T Occurrence of reo-like viruses in young children with acute gastroenteritis. Diagnoses established by electron microscopy and complement fixation, using the reo-like calf virus as antigen. Acta path. microbiol scand. Sect. B, 84 22–28 1976.

In the course of a six-month-study of acute gastroenteritis in children of ages up to six years, a reo-like virus was found in 54 per cent of the faecal specimens obtained at an early stage of the disease, using electron microscopy as screening test. By means of a concentrated complement fixation antigen, composed of a related calf diarrhoea virus cultivated in tissue culture, the rise in titre was found to be significant in 96 per cent of the patients whose faeces contained the reo-like virus. Antibodies were present in the remaining 4 per cent without rise in titre. In 10 per cent of the cases with gastroenteritis infection was caused by adenovirus or Salmonella. A probable aetiological agent was found in 71 per cent of the patients. It applies to 33 per cent of all cases caused by the reo-like virus that they were nosocomial infections.

Key words Reo-like virus gastroenteritis complement fixation.

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Enteropathogenic bacteria enterovirus and adenovirus are implied only in about 10–20 *per cent* of the cases of gastroenteritis in young children (4 13) Until recently the causative agent in the remaining cases has been unknown. The occurrence of reo-like virus in faeces and duodenal mucosa of patients with acute gastroenteritis has lately been described by *Bishop et al.* (2, 3) Findings of a similar virus in faecal samples from patients with acute gastroenteritis were at the same time reported by *Flewett et al.* (6 7) The incubation period of acute gastroenteritis caused by this virus has been estimated to range from 24 to 48 hours (4) excretion of virus will usually continue for one week after onset of illness (4 7) Morphologically identical viruses have lately been reported by several investigators in other countries (1 5 9 11 15 16) The virus reminds morphologically of the reoviruses, but can be distinguished from these both serologically (7 8, 9) and by its fine structure (2, 7 8) It has been shown by immunoelectron microscopy (IEM) and complement fixation (CF) that

the Nebraska Calf Diarrhoea Virus (N.C.D.V.) and the virus of epizootic diarrhoea of infant mice (E.D.I.M.) serologically are related to the human virus (8, 9). So far electron microscopy (EM) has been the only method to identify the virus. The diameter of the virus is 67 nm. It consists of a core of 38 nm and two capsomer layers on top of each other (6, 7). *Flewett et al.* suggested to apply the term rotavirus to these reo-like virus, while *Bishop et al.* proposed duovirus (8, 4). Attempts have been made to culture the human virus, but so far it has only been possible to make it grow out on organ cultures of foetal human intestinal cells (15). The calf virus, however, can be cultured on calf kidney cells (12, 14).

The aim of this work was to survey the incidence of acute gastroenteritis caused by this virus among children seen in the paediatric clinic of Malmö General Hospital. Furthermore, utilizing the serological relationship to N.C.D.V., the present authors intended to develop a CF-test to be used in diagnostics.

PATIENTS AND METHODS

Patients. During the period from November 1st 1974 to April 30th 1975 114 children of ages up to six years ere admitted to hospital on the indication of gastroenteritis; this group represents about half of the total number of patients in this ge group with gastroenteritis admitted to Malmö General Hospital during this period. A control group of 29 children in the same age group admitted to the hospital during the same period but under other diagnoses, was included in the study though the group was not directly matched to the gastroenteritis patients. The group of patients with gastroenteritis comprised 61 male and 53 female patients, the control group comprising 17 males and 1 females. It applies to both groups that the majority of patients were in the age group 6–18 months.

Faeces. Faecal samples were obtained from all patients immediately after admission to hospital as rule within one week fter onset of illness, and from some patients also t later stages of the disease with a ew to control the duration of irus excretion. Faecal extract were negatively stained and examined by EM, as described by *Flewett et al.* (6, 7). Virus finding were graded according to … + scale. All faecal samples were also examined for Salmonella and Shigella, while no attempts were made to examine for enteropathogenic *E. coli.*

Sera. As a rule, sera were obtained from patients 1–3 days after onset of illness and also 2–3 weeks later. Paired sera were obtained from 85 patients with gastroenteritis and from 23 patients in the control group.

Tissue cultures. All faecal specimens were cultured on green monkey kidney cells, vero cells and on local HeLa-like strain, according to the routine technique used in the laboratory. The calf virus was cultured on calf kidney cells using Eagles maintenance medium without foetal calf serum at maximum of 15 days, the medium being changed every 5th day. The occurrence of virus was checked by EM as only a partial cytopathogenic effect was visible. Media and cell suspensions containing virus were pooled and stored t −20 C.

IEM. Three paired sera from patients with gastroenteritis and reo-like particles in faeces and a rabbit antiserum to the N.C.D.V. were tested against clarified human faecal extract containing reo-like viruses and against suspension of the corresponding calf virus according to a method described by *Flewett et al.* (8). The results were read as percentage of agglutinated particles.

CF-test. The Lincoln strain isolated by dr. *C. A. Mebus*, Department of Veterinary Science, University of Nebraska, USA, during an outbreak of N.C.D.V. was kindly supplied by dr. *A. Meyling*, Copenhagen, and used as CF-antigen. The pooled media were frozen and thawed three times. The fluid was clarified and virus was pelleted by centrifugation according to the same method as that used for faecal extracts. The pellet was resuspended in 0.1 M veronal buffer to one tenth of the original olume. The antigen had a titre of about 1/32 in chessboard titration and two units of antigen were used in the test. All paired sera were tested against the Lincoln antigen. Paired sera which failed to show a significant rise in titre against the Lincoln antigen were tested against adenovirus and enterovirus (Coxsackie B5).

RESULTS

Virological and serological findings. The result of the examination by EM is listed in Table 1. Reo-like virus was found in 61 out of the 114 cases (54 per cent) of gastroenteritis examined. The same test of all children in the control group was negative. Adenovirus and enterovirus occurred in 19 cases (17 per cent) in the gastroenteritis group and in 4 cases (14 per cent) in the control group. Salmonella was found in 3 cases (2

Table 1 *Enteric Pathogens in Faeces from Children with Acute Enteritis and from Children in a Control Group Demonstrated by Electron Microscopy and Bacterial Cultivation*

Enteric pathogen	Children with acute enteritis (114)	Control children (29)
Reo-like virus	61 (54 %)	0
Adenovirus	15 (13 %)	1
Enterovirus	4‡ (3 %)	3
Salmonella	3 (2 %)	0
Negative	38 (33 %)	25

* In four cases, adenovirus occurred together with reo-like virus.

‡ In three cases, enterovirus occurred together with reo-like virus

per cent) in the former group In 7 cases (6 per cent) adenovirus or enterovirus was found together with reo-like virus. In 5 cases, in which the excretion of reo-like virus was followed in repeated samples, virus was demonstrable up to eight days after onset of illness, except in one case in which virus was excreted 14 days after onset of illness. There was a peak in excretion 3–4 days after onset of illness. No reo-like virus could be isolated in tissue culture, while adenovirus was isolated in 8 out of the 15 cases, in which adenovirus was found in faeces by EM. It applies to all four enterovirus that the findings by EM could be confirmed by isolation in tissue culture.

The IEM revealed a close relationship between the calf virus and the human virus. As shown in Table 2, all human virus particles were agglutinated by human convalescent sera, while acute sera did not agglutinate the virus. The calf virus was agglutinated to about 80 per cent by the same human convalescent serum. The rabbit antiserum to N C.D V agglutinated the human virus as well as the calf virus in 100 per cent.

Forty nine paired sera were available from the 61 patients who by EM had been found to be positive. Forty-seven (96 per cent) showed a significant rise in CF titre against the N C.D V (Table 3) while antibodies were present in the two remaining paired sera without rise in titre. The first samples of serum were not drawn from these two patients until one week after onset of illness. Among the patients in whom EM failed to demonstrate reo-like virus in faeces a fourfold or greater response was detected in four patients. Another 5 cases were positive but samples of faeces were not obtained. In the control material comprising 23 paired sera there was no significant rise in titre.

In order to save sera, only a limited number of paired sera were tested in CF against adenovirus. One of the four patients with

TABLE 2. *Agglutination by Different Sera of Reo-like Virus from Man and Calf using IEM*

Patient	EM of faeces*	Agglutination (per cent)		
		Serum sample	Human virus	Calf virus
M.L.	+ + + +	acute	—	—
		conv ‡	100	80
H.B.	+ +	acute	—	—
		conv ‡	100	85
H.R.	+ + + +	acute	—	—
		conv ‡	100	85
Rabbit 25 (inoculated with calf virus)	—	Hyperimmune serum†	~ 100	100

* findings of reo-like virus graded + to + + + +

‡ dilution 1/5

† dilution 1/10

TABLE 3 *Frequency of Complement Fixing Antibody against NCDV in Acute Gastroenteritis and in a Control Group*

	Number of paired sera tested	Antibodies			
		Significant rise in titre	No rise in titre	No antibodies	Not tested
Patients with gastroenteritis, reo-like virus in faeces (EM)	49	47	2	0	12
Patients with gastroenteritis, no reo-like virus in faeces (EM)	28	4	6	18	17
Patients with gastroenteritis, samples of faeces not available	8	5	0	3	—
Control patients	23	0	11	12	6

convalescent sera not available

gastroenteritis, in whom both reo-like virus and adenovirus were found, showed a fourfold or greater antibody response, while 5 out of 9 patients with gastroenteritis in whom only adenovirus was present in faeces, presented a significant rise in titre against adenovirus. In 7 out of 17 patients with gastroenteritis by EM found to be negative and without significant rise in titre against the N.C.D.V., a fourfold antibody response against adenovirus could be demonstrated. No rise in titre against this antigen was observed in sera from patients in the control group. In one out of the four patients with gastroenteritis in whom enterovirus was found, a significant rise in titre against the Coxsackie B5-antigen was demonstrated.

In the series studied, acute gastroenteritis was most commonly seen in January (Fig. 1). Reo-like virus was the pathogen most commonly seen throughout the year. The total number of positive cases was highest in January while the percentage increased during the Spring months. The number of cases in which adenovirus was demonstrable also reached a peak in January. The distribution of infections in relation to age of patients is illustrated in Fig. 2. Most of the patients with gastroenteritis belonged in the age group 17 months and the same applies to most cases in which gastroenteritis was associated with a presence of the reo-like virus. The youngest child found to be positive was 20 days old, the oldest was 4 years old. Occurrence of adenovirus showed the same age distribution as reo-like virus.

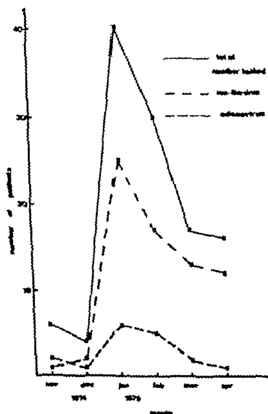


Fig. 1 Distribution over months of patients excreting reo-like virus and adenovirus and/or presenting significant rise in titre against these viruses in relation to total number tested.

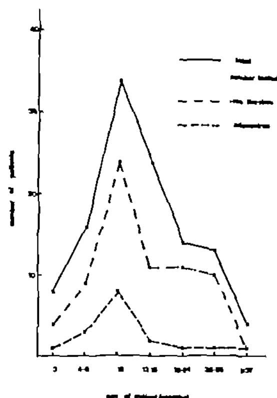


Fig 2 Relation of reo-like irus and adenovirus *excretion and/or significant rise in titre against* these viruses to age of patients.

Twenty-two (33 per cent) of the patients with gastroenteritis and reo-like virus in faeces and/or a significant rise in titre against the N C.D V originally received treatment based on diagnoses other than gastroenteritis. Gastroenteritis developed in these patients either when they had stayed in the childrens ward for more than 48 hours, or within 24 hours after they had returned home. Fourteen (64 per cent) of these patients were less than one year old.

Clinical findings The symptoms and their duration in 67 cases of gastroenteritis associated with the presence of the reo-like virus, diagnosed by EM and/or CF are listed in Table 4 Diarrhoeas would usually persist for about 4 days. The temperature was elevated in 72 per cent of the patients, the fever lasting for 2–3 days and averaging 38 7 C. Gastroenteritis did not run a fatal course in any case during the period of this study One

TABLE 4 *Clinical Findings in 67 Cases of Acute Gastroenteritis Caused by R o-like V irus*

	Number	%	Median duration, in days
Diarrhoea	67	100	4 (1–14)
Vomiting	56	84	3 (1–6)
Fever (≥ 37 .8)	48	72	2–3 (1–6)

* median temperature 38 7 (37 .8–40°.2)

patient was moribund at the time of admission A further four were dehydrated to such a degree that intravenous drop was required. In general, however the patients were not severely ill.

DISCUSSION

Reo-like virus could be demonstrated by EM in 54 per cent of all cases of gastroenteritis studied while this virus was not found in faeces from patients in the control group, thus indicating an association between the virus and the disease The occurrence of virus in patients with gastroenteritis was most common during the colder season, a peak being seen in January This, as well as the fact that virus is most commonly seen in children in the age group 7–18 months is in agreement with findings in previous studies (4 7 15) A further evidence of an association between the virus and the disease is the occurrence of increasing titre against the virus in paired sera. This was shown simultaneously by *Flewett et al* (8) and *Kapikian et al* (9) using IEM. The observations could be confirmed by the present authors in three paired sera from patients with gastroenteritis. *Kapikian et al* (9) used faecal extracts as antigen in a CF test and found a significant rise in titre in paired sera from EM positive cases of gastroenteritis. By this method it was also possible to demonstrate the antigenic relation to the N C.D V In the present study where concentrated N C.D V was used as antigen, a fourfold or greater serological response could be shown in 47 out of 49 (96 per cent) cases of gastroenteritis. The results obtained

TABLE 5. *Probable Aetiology in 114 Cases of Acute Enteritis in Young Children*

Reo-like virus	faeces positive (EM)	61	(61.5 %)
	faeces negative (EM) positive CF	4	
	faeces not available positive CF	5	
Adenovirus	positive CF	7	(6.0 %)
Enterovirus	positive CF	1	(1.0 %)
Salmonella		3	(2.5 %)
Total positive		81	(71.0 %)
Negative		33	(29.0 %)

by the present authors by EM could be almost completely confirmed serologically by CF using a N.C.D.V.-antigen. Thus correlation between EM and CF is closer than that recently reported by *Kapikian et al.* (10) who only in about two thirds of the verified cases of gastroenteritis caused by reo-like virus could show rising titre against another strain of the N.C.D.V. In the present study the four cases found to be positive by CF but negative by EM, can be explained by the fact that the faecal samples from these patients were collected rather late in the course of the disease, more than one week after its onset. Taking into account the five cases from which faecal specimens were not obtained, the number of cases of gastroenteritis associated with reo-like virus totalled 70 out of 114 (62 per cent) in the present study (Table 5). Considering the incubation time, it is obvious that one third of the patients in whom the reo-like virus was the causative agent were contaminated during their stay at the hospital. If the 7 cases in which the titre against adenovirus increased, as well as the case involving enterovirus and the 3 cases involving Salmonella are included a probable aetiology could be shown in 81 out of 114 cases (71 per cent).

EM is a very rapid and convenient method by which to demonstrate the reo-like particles, but an expensive equipment is required. In laboratories not equipped with an electron microscope the CF test used in the present study is another possibility even if the diagnoses cannot be established in the early stage of the disease. CF can also be used for epidemiological studies. From a clinical point of view it is urgently required to have the diagnosis established as the disease may be of severe nature. Thus, *Middleton et al.* (13) and *Bishop et al.* (4) have reported 7 and 5 fatal cases, respectively. In the present study there were no fatal cases. Still, one patient was in very poor condition at the time of admission.

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HUMAN ECTO- AND ENDOCERVICAL EPITHELIAL CELLS AS TARGETS FOR HERPES SIMPLEX TYPE TWO INFECTION *IN VITRO*

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Vesterinen, E. Human ecto- and endocervical epithelial cells as targets for herpes simplex type two infection *in vitro*. Acta path. microbiol. scand. Sect. B 84: 29–37, 1976.

An in vitro method was used to compare the herpes simplex virus type 2 (HSV_2) susceptibility of human ecto- and endocervical epithelial cells as well as the induced cellular alterations. HSV caused a productive infection and morphological alterations both in ecto- and endocervical epithelial cells, but the cytopathogenicity, virus production and development of HSV_2 antigens showed a greater sensitivity of ectocervical cells to HSV_2 infection. The cytopathogenicity and production of infectious virus did not depend on the antibody pattern of the target cell donor patients. Basically similar morphological alterations were seen in ecto- and endocervical cultures and the various morphological types of altered cells are described.

Key words: Cervical epithelial cells; herpes simplex.

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Herpes simplex virus (HSV) is a fairly common inhabitant of the female reproductive system (10, 20). Because of the possible association of this virus with cervical carcinoma (16) and the potential risk of maternal herpes infection of the newborn infant (14) this virus has attracted considerable attention as an important infective agent of the female genital tract.

HSV often causes clinically inapparent infection which can be quite accurately detected by the cervical Papanicolaou smear (10, 11, 23). Although patients with cervical carcinoma have a higher incidence of HSV antibodies than controls (15), sero-epidemiological investigations alone cannot resolve the role of the HSV in cervical carcinoma. *Duff & Rapp* (6) have been able to induce malignant transformation of newborn hamster cells infected *in vitro* with UV-inactivated HSV_2, and *Darai & Munk* (4) observed a morphological transformation of human embryonic lung cell cultures infected with non-irradiated HSV_2.

Human ecto- and endocervical epithelial cell cultures have proved to be a suitable system in investigations of the cytopathogenicity of cytomegalovirus to epithelial cells (22). The present study was undertaken to develop an *in vitro* method by which to investigate effects of HSV_2 infection directly

TABLE 1 *Proportion of HSV Infected Explants l c ding to l rus Dose Showing Cytopathic Effect in Human Ecto- and Endocervical Cultures (Infected 7 Days after Explantation)*

Post infection time (h)	Ectocervical cultures 1 × 10: No. of ltures	No. of altered explants	1 × 10: No. of ultures	No. of altered explants	1 × 10²: No. of cultures	No. of altered explants	Endocervical cultures 1 × 10: No. of cultures	No. of altered explants	1 × 10²: No. of cultures	No. of altered explants	1 × 10: No. of cultures	No. of altered explants
7	3	19/19‡ (+)					3	0/13				
20	4	16/16 (++)	1	3/3 (+)			3	8/12 (+)				
24	4	12/12 (+++)	3	7/7 (++)			3	12/12 (++)	2	1/8 (+)		
32	3	13/13 (+++)	2	5/5 (+++)	1	1/4 (+)	2	6/8 (+++)	2	6/8 (+)	1	0/4
48	3	10/10 (+++)	2	3/3 (+++)	1	4/5 (++)	2	4/5 (+++)	2	3/3 (++)	1	1/3 (+)

Virus grown in MRC-5 cells. The input dose is indicated in $TCID_{50}$ titre in each column heading.
‡ Grade of morphological alterations as shown in Papanicolaou stained preparations: + = not more than 1/4 of the area of explants showed CPE; ++ = 1/4–1/2 of the area of explants showed CPE; +++ = over half of the area of explants showed CPE. The cultures were kept under observation and fixed and stained at the indicated time intervals after the first signs of alterations.

Table 2 *Number of Cytopathic Foci* in Ecto- and Endocervical C ltures‡ after I fection with HSV₂† in vitro (in Explants from a Patient with "Intermediate" Type Antibodies and in Secretory Phase of Menstru l Cycle)*

Post-infection time (h)	Ectocervical explants Average No. of foci per low power field (2.5 ×)	Endocervical explants Average No. of foci per low power field (2.5 ×)
7	1	0
14	5	1
20	8	2
24	>10$	4
32	>10$	7
96	ↄ	ↄ

* CMC overlay used
‡ Three ecto- and 3 endocervical cultures, average area of explants = 1–2 low power fields per culture.
† Virus input 1×10^4 $TCID_{50}$.
$ Foci fused over large areas.
ↄ Explants destroyed.

CPE, while all ectocervical explants were destroyed to variable degree. Also with lower input multiplicities, this delay was evident in the onset of CPE in endocervical cultures (Table 1) Similar results were obtained when cultures infected 14 days after explantation were investigated (data not shown)

HSV foci in ecto- and endocervical explants Five ectocervical and five endocervical cultures initiated from three patients were covered after inoculation with 0.5 per cent CMC in growth medium and the susceptibility of the explants was determined by counting the number of foci showing CPE. Examined under inverted microscope (Leitz, Wetzlar Germany) the number of foci per low power field (objective 2.5 ×) was greater in ectocervical than in endocervical monolayers (Table 2) In endocervical explants the foci tended also to be smaller and consisted of fewer altered cells.

In five ecto- and endocervical cultures, the number of infective foci was examined by immunofluorescent staining (Table 3) Also by this technique, a greater number of foci per low power field (a Leitz, Ortholux microscope with a HBO 200 mercury vapour lamp and Balzer s FITC interference filters, objective 10× was used) were detected in the ectocervical monolayers.

Fluorescent antibody stained cells in ectocervical cultures presented already after 7 h an intensive cytoplasmic fluorescence which in most cells was predominantly perinuclear (Fig 1) At this time some cells in endocervical explants had faintly fluorescing intranuclear granules and a weak perinuclear fluorescence was detectable only in high power magnification and considered to be a sign of an early phase of HSV_2 replication (18) After 10 hours' infection, strong perinuclear fluorescence was seen also in some endocervical cells (Fig 2) but at this time, this fluorescence pattern was already dominating in the ectocervical cells. A few ectocervical cells presented intensive fluorescence throughout the cell, representing late phase of the viral replicating cycle. Cells presenting fluorescence were not detected in uninfected cultures.

Production of infective virus by ecto- and endocervical explants Infective virus was assayed from the supernatants of ecto- and

TABLE 3 *N mber of HSV Foci as Detected by Imm nofluorescenc in E to- and Endocervical Explants* after Inf ction in vitro‡*

	Post infection time (h)	Average No. of foci per low power field (10×) Ectocervical explants	Endocervical explants
Donor patient A.R.†	8	5	1
Donor patient K.S $	7	4	1
	10	>10ↄ	6

* Average area of explants = 7–10 low power fields (10×) per culture
‡ Explants infected 7 days after explantation.
† Three ecto- and 3 endocervical cultures, virus input 1×10^4 $TCID_{50}$.
$ One ecto- and one endocervical culture in both experiments, virus input 1×10^7 $TCID_{50}$.
ↄ Foci fused over large areas.



Fig 1 Photomicrograph of HSV_2-specific perinuclear fluorescence in HSV_2 infected ectocervical epithelial cells. The indirect immunofluorescence technique was used (7 h after infection) × 125

endocervical explants at various time intervals (Table 4) Infective virus could not be detected earlier than after intervals of 32 h in either ecto- or endocervical cultures, but the supernatants from ectocervical cultures contained considerably higher titres of infectious virus. In both cultures, the amount of infective virus decreased significantly later in the course of infection.

Cellular morphology induced by HSV The morphological alterations detected in the nuclei and cytoplasm were grossly similar in HSV infected ecto- and endocervical cells. In ectocervical explants, the first changes were mostly seen at the explant margins Chromatin fragmentation and small basophilic inclusions appeared in the nuclei and cell fusion was detected (Fig. 3) In places, nuclear homogenization and chromatin margination gave a "ground glass" appearance to the altered nuclei (Fig 4) As the destruction of explant colonies spread, the number of multinucleated giant cells increased. The same phenomenon was seen also in endocervical explants (Fig 5) though to a lesser degree. Large eosinophilic or basophilic intranuclear inclusions, surrounded by a more or less prominent halo (Fig. 6) were detected both in ecto- and endocervical epithelial cells. In endocervical cells, the inclusions usually were distinct and regular whereas in the ectocervical cells they were somewhat irregular and diffuse. Inclusion bearing cells were mostly seen in an advanced stage of the infection. This was also demonstrated by the fact that these cells were seldom seen in colonies where the integrity was preserved, and the proportion of inclusion-bearing cells increased with advancing destruction of explant colonies. Marked nuclear vacuolization was often present. The cytoplasm of HSV_2 altered cells was veil-like, opaque and eosinophilic or basophilic (Fig.

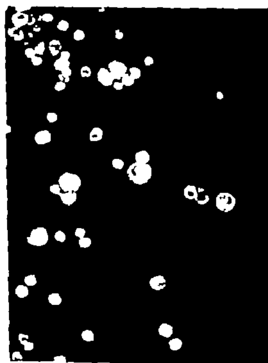


Fig 2 Perinuclear immunofluorescence in HSV_2 infected endocervical epithelial cells (10 h after infection) × 125

TABLE 4 *Production of Infectious Virus in HSV * Infected Ecto- and Endocervical Epithelial Cell Cultures*

Post-infection time (h)	Donor patient E.A.‡		Donor patient K.S.†	
	PFU/ml$ of supernatants from ectoc. explants	PFU/ml of supernatants from endoc. explants	PFU/ml of supernatants from ectoc. explants	PFU/ml of supernatants from endoc. explants
0	0	0	0	0
7	0	0	0	0
20	0	0	0	0
27	0	0	0	0
32	0.32×10^3	0.02×10^3	1.19×10	0.04×10^3
54	0.07×10^3	0	1.34×10^3	0.02×10^3
72	0	0	0.26×10^3	0

* Virus input 1×10^4 $TCID_{50}$

‡ Antibody pattern compatible with type 2 antibodies secretory phase of menstrual cycle. Average area of ectocervical explants = ½ low power field (2.5×) Average area of endocervical explants = 1 low power field (2.5×)

† Antibody pattern of "intermediate" type, proliferative phase of menstrual cycle. Average area of ectocervical explants = 1 low power field (2.5×) Average area of endocervical explants = ⅓ low power field (2.5×)

$ Plaque forming unit/ml.



Fig 3 Early stage of herpetic infection in an ectocervical explant. Cell fusion present without marked nuclear or cytoplasmic alterations. Papanicolaou stain × 350

5) In ecto- and endocervical control cultures no changes compatible with HSV altered cells could be seen although some cultures were kept under observation for up to three weeks.

When cultures initiated from 9 patients with HSV antibodies and from 2 patients without antibodies were compared, "ground glass" nuclear morphology as well as intranuclear inclusions were seen in the altered cells in the same explants irrespective of the antibody pattern of the donor patients.



Fig 4 Multinucleated giant cell with "ground glass" nuclear morphology. Endocervical culture, Papanicolaou stain × 850.



Fig 5 HSV altered endocervical epithelial cells with predominantly "ground glass" nuclear morphology Papanicolaou stain × 600

The phase of the menstrual cycle of target cell donor patients did not have any notable influence on the cytopathogenicity of HSV_2 to ecto- and endocervical cultures.

DISCUSSION

The present results show that herpes simplex virus type 2 (HSV) causes a productive infection with cytopathic effect (CPE) both in ecto- and endocervical epithelial cells *in vitro* while ectocervical cells are found to be significantly more sensitive. In ectocervical cells with different virus inputs, CPE appeared constantly earlier than in endocervical monolayers. At high input multiplicity all ectocervical explant colonies showed alterations already after 7 h whereas only 50 per cent of the endocervical explants showed cytopathic effect after 20 h. The number and size of foci were also greater in ectocervical monolayers in cultures where the spreading of virus through medium was prevented by carboxymethylcellulose In fluorescent antibody stained preparates, the HSV antigen positive cells were more frequent in ectocervical cultures. In ectocervical explants, marked perinuclear immunofluorescence was seen in many cells already 7 h after infection, whereas only faint perinuclear and granular intranuclear fluorescence at this time was detectable by high power magnification in endocervical cells. Ectocervical explants also yielded more infective virus and all these data indicate that the infective cycle in ectocervical cells is more effective and rapid than that in endocervical cells.

The explanation of the difference in sensitivity of these two cell types remains obscure. The possible antiviral effect of cervical mucus was eliminated by careful washing of cultures before inoculation. The synthesis of HSV_2 in endocervical cells may be slower or the adsorption of HSV_2 to endocervical cell surface may be hindered by a deficiency of specific receptors. The situation is the reverse if the explants are infected with cytomegalovirus (CMV) the endocervical cells being more susceptible to CMV infection than the ectocervical cells (22) The same situation is encountered in cervical smear preparations where HSV altered cells are found more often on the ectocervical part of the specimen



Fig 6. Giant multinucleated ectocervical cell with molded nuclei and prominent multinuclear inclusions, surrounded by halo. Papanicolaou stain × 850

during HSV infection (23) whereas the few cases of CMV altered cells documented have been found only in endocervical (7) or endometrial (5) biopsy specimens.

The order of development of the different morphological changes seemed to be the same in ecto- and endocervical explants. The first cellular alterations in HSV_2 infected cultures regularly consisted of cell fusion, chromatin fragmentation and small intra nuclear basophilic inclusions (Fig 3) Along with the destruction of explant colonies, cells with "ground glass" nuclear appearance and with intranuclear large inclusions appeared in cultures. Intranuclear inclusion-bearing cells were seen in larger proportions in cultures with prominent CPE, indicating a late phase of HSV replication.

Irrespective of the HSV antibody pattern of the target cell donor patients, the response of explants to HSV_2 infection was similar as to extent of morphological changes and capacity to yield infectious virus. This could lead to the assumption that the cervical epithelial cells do not harbour latent HSV_2. In the work by *O Neill et al.* (13) HSV latency was induced *in vitro* by cytosine arabinoside. These cells were susceptible to superinfection but only a small proportion of cells actually harboured the virus. *Stevens et al.* (19) were able to induce latent spinal ganglionic HSV infection in mice by inoculation into the footpads of hindlegs *Waltz et al.* (21) were able to repeat this by vaginal HSV inoculation. Latent HSV infection seems to be possible also in local sensory ganglia in man, as reported by *Bastian et al.* (3) In the present work, some control cultures were observed for up to three weeks and, within this period, no signs of HSV infection could be seen although the cultures were initiated from HSV_2 seropositive patients. In a recent study by the present author (23) concerning cytologically diagnosed gynaecological HSV infections, the morphological alterations were similar at the first and later detections of the infection in all cases. Thus, it may be misleading to draw conclusions about the primary or recurrent character of gynaecological HSV infection on the basis of cytological aspects only The different morphological types of altered cells found during HSV infections in PAPA smears (1 12) or in HSV_2 infected cervical organ cultures (2) would rather seem to represent the stage of HSV replication of an overt infection at the moment the sample is obtained regardless of the seroepidemiological status of the patient.

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OSMOTICALLY INDUCED CHANGES OF CELL SPACES IN *NEISSERIA MENINGITIDIS* COMPETENCE VARIANTS

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Jyssum, K. Osmotically induced changes of cell spaces in *Neisseria meningitidis* competence variants. *Acta path. microbiol. scand. Sect B, 84 38–44 1976*

The volume of the whole cell and the fraction of the intact cell bounded by the cytoplasmic membrane (protoplast volume) have been measured by dextran and C-sucrose exclusion spaces in *Neisseria meningitidis* competence variants. Increase in external osmotic pressure causes contraction of the protoplast volume. Increasing osmolality due to NaCl and $MgCl_2$ also causes contraction of the volume of the whole cell, whereas increasing concentrations of sucrose cause little or no change in the whole cell volume. The experiments demonstrate a significant difference between competent (*p*) and incompetent (*cp*) cells. The *p* protoplast have a far higher capacity for swelling during decreasing osmolality and for shrinkage during increasing osmolality Comparison of spheroplasts obtained by autolysis as well as by the penicillin technique indicates that the average *cp* spheroplast is larger than the average *cp* one. The significance of the difference in structure of *p* and *cp* protoplasts has been discussed

Key words *Neisseria meningitidis* competence variants cell spaces osmosis

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Competent (*cp*) variants of the *Neisseria meningitidis* Strain M1 have been found to be much less sensitive to osmotic stress than incompetent (*cp*) ones. Thus, the exposure of the cells to increased osmotic pressure as well as to hypotonic conditions leads to higher lethality and more pronounced membrane damage in the incompetent variant (4) The subsequent consequences of osmotic stress such as induction of autolysis of the cell walls (5) and cell membranes (6) are also more pronounced in this variant.

It was postulated that the essential difference between the competence variants might be in the realm of membrane structure or function (4) But the difference does not seem to be due to a higher tensile strength of the membrane of the competent variant since the competence variants were equally fragile as determined by the osmotic strength necessary for stabilization of spheroplasts (6)

Increase in turbidity of *N meningitidis* cells due to the addition of various salts and sucrose is strictly dependent on the osmotic pressure, after correction for the changes in medium refractive index (4) But it seems that the increase in turbidity under hyper osmotic conditions, and under equal osmolality is regularly higher in the competent variant. This may indeed be an indication that this variant is more free to shrink upon exposure to hypertonic conditions than the incompetent one. It was suggested that this could mean that the *cp* variant is provided with membrane invaginations or true mesosomes. This would provide the *cp* cell with an extra protection against the harmful effects of shrinkage (plasmolysis) and swelling (plasmoptysis) (4 5 6)

The present experiments were designed to explore the possibility whether the protoplasts of competent variants of *N meningitidis* due to the existence of cytoplasmic invaginations might be larger than those of incompetent ones. The paper first reports some measurements of the cell and protoplast volumes of the N *meningitidis* competence variants at various osmotic pressures. Data concerning the relative size of autoplasts and penicillin spheroplasts from the competence variants are also presented.

MATERIALS AND METHODS

Many of the media and manipulations used in this study have been described in preceding papers concerned with the influence of osmotic pressure on *N meningitidis* competence variants (4 5 6) Some procedures which have been modified, and some manipulations and calculations pertinent to the present work are listed below

Bacteria The wild type *N meningitidis* Strain M1 was generally used along with the auxotrophic mutant M1-6 *his pr* and M1-8 *his arg* Competence in transformation was indicated by the symbol *p* Growth requirement and competence in transformation were controlled as described previously (4 5 6)

Measurement of cell spac s. The capacity of sugars and glycols to penetrate the cell wall increases parallel with reducing size. Polyglycols or dextrans of molecular weight above 10 approximately are unable to penetrate the cell wall of *Bacillus megaterium*. Molecules smaller in size than stachyose are able to penetrate the cytoplasmic membrane (9) Experiments with *B. megaterium* (9) and *Escherichia coli* (1) have shown that it is possible to measure whole cell exclusion spaces using Sephadex marker blue dextran 2000 of an average molecular weight of 2×10^6 Stachyose can be used to determine the protoplast volume but the slightly smaller sucrose can be used instead of the expensive stachyose, in all essentials the results will be the same (1)

Cell spaces in the N meningitidis competence variants were measured by the f llowing technique which is a slightly modified form of that used by *Alemohammad & Knowles* (1) The competence variants were grown in batch cultures in HIB (Heart Infusion Broth Difco) and harvested during exponential growth, unless otherwise noted (4 5 6) Suspensions were prepared in 10 mM imidazol buffer (Imidazol-buffer Sigma pH 6.8) supplemented with 0.1 M N Cl. The concentration of cells was such that 18 ml contained 1.2–1.8 g wet weight. An 18 ml portion was dded to a tared centrifuge tube and centrifuged at 38000 × g for 20 min at 4 C. The supernatant was discarded and the inside of the tube was wiped with a tissue and then re-weighed. The following was then added to the pellet: 1) Marker blue dextran (Blue dextran, average molecular weight 2000000 Sigma) 0.5 ml 2 per cent W/V in 10 mM imidazol buffer 2) Uniformly labelled ^{14}C-sucrose, 0 1 ml (to give a final concentration of 3 mM and 50000 to 100000 cpm.) 3) Imidazol buffer 10 mM 0.5 ml with sol te to give the final osmolality desired. The solutes were either NaCl, MgCl or sucrose, as indicated in the individual experiment. The contents were carefully mixed in the tube After 10 min at 4 C, the mixture was centrifuged at 38000 × g for 20 min at 4 C, and the supernatants were removed. The sucrose concentration was measured by the addition of 0 1 ml of the supernatant to 10 ml scintillation fluid (1 part triton-X 100 was mixed with parts of a solution containing 5 g PPO and 0.05 g dimethyl POPOP per 1 toluene) and counting in a Packard tricarb liquid scintillation spectrometer The dextran concentration was assayed by measuring suitable dil tion at 620 nm in the Beckman DB spectrophotometer Each experiment was done in triplicate. Control dilutions of the ^{14}C-sucrose and dextran were done exactly as above using 18 ml buffer only

Calculation of cell spaces. The calculations followed the principles outlined by *Black & Gerhardt* (2) The space (or fraction) of the pellet penetrated by the sol te Sw can be evaluated from the equation

V being the volume of the added ^{14}C-sucrose and dextran while C_0 and C_f represent initial and final concentrations, respectively W_p indicates the weight of the pellet. Since the density of a suspension of *N meningitidis* (like that of *E. coli*) is close to 1 weight measurements are essentially an index of volume.

The weight of the cells (which is essentially the same as volume) can be obtained from the equation

$$W_{(cell)} = W_p\,(1 - S^{w}_{dextran}) \qquad (2)$$

and that for the protoplast from

$$W_{(protoplast)} = W_p\,(1 - S^{w}_{sucrose}) \qquad (3)$$

Table 1 shows a typical experiment comparing cell spaces in the competence variants as measured with 0.05 M and with 0.6 M NaCl (approximately 2 1 atmospheres and 24 7 atmospheres osmotic pressure)

Penicillin spheroplasts Batch cultures of 12 ml HIB were inoculated from an exponentially growing culture in the same medium and grown from A approximately 0 100 to A around 0.300 at 37 C on a reciprocal shaker The cells were harvested in the centrifuge (2500 × *g* for 10 min) and resuspended in HIB with 0 7 M sucrose and 0.05 M $MgCl_2$ ("spheroplast broth) After resumption of growth (5) Penicillin G 100 µg/ml was added. The culture was incubated during shaking for 2 hours, and then left t stand at 37 C. After their release (6) the spheroplasts were spun down in the centrifuge Suspensions were prepared in 0 7 M sucrose with 0 1 M Na-acetate and 0.05 M $MgCl_2$ *and kept in a humid chamber at 20 C.*

Autoplasts. The *N meningitidis* competence variants were grown in 12 ml batches of HIB, harvested and resuspended in the spheroplast broth as described for the preparation of penicillin spheroplasts. Resumption of growth in the "spheroplast broth" was followed by reading of the absorbancy After 2 hours, *the cells were harvested in the* centrifuge (2500 × *g* for 10 min) and the pellet from 24 ml was resuspended in 2 ml containing 0 1 M Na-acetate, 0 7 M sucrose and 0.05 M $MgCl_2$ (6) The suspensions were left t 20° C. The development of spheroplasts took place during the succeeding 20-30 hours followed by phase contrast microscopy (6)

Comparison of spheroplast size Spheroplasts from the competence variants were compared by phase contrast microscopy Samples were prepared from the spheroplast suspensions and studied in Reichert microscope with phase contrast equipment, as previously described (5) Photographs were taken using Kodak Tri-X Pan black and white film t a final enlargement of 1000 × The Reichert Photo-Automatic equipment (model 6A D/67) was used for the photography Usually each film (36 exposures) contained 18 exposures from the *p* spheroplasts and 18 exposures from the *cp* spheroplasts that were to be compared. The pictures of the spheroplasts were projected on a screen (lens with *f* = 10 cm 1 2,8, and at a distance of 2 m) and the diameters of the spheroplasts were measured with callipers. Ten spheroplasts randomly chosen from each microscope slide were measured One experiment usually compared spheroplasts in 4 slides from *cp* cells and 4 slides

TABLE 1 *Experimental Dat and Calculations f Cell Spaces* N meningitidis *Competence Variants*

	NaCl concentration			
	0 05 M		0 6 M	
	p	*p*	*cp*	*cp*
Pellet weight W_p (g)	1 4344	1.3925	1.0615	1 1724
Initial sucrose conc. C_0 (cpm)	97130	97130	96753	96753
Initial dextran conc C_0 (A_{620})	0.268	0.268	0.243	0.243
Sucrose conc. C_f (cpm)	54069	45880	49130	47420
Dextran conc. C_f (A_{620})	0.219	0 183	0.202	0 173
Sucrose space $S^{w}_{sucrose}$	0.4996	0.6313	0.8218	0 7986
Dextran space $S^{w}_{dextran}$	0 1403	0 2625	0.1721	0 3106
Protoplast volume $W_{protoplast}$	0 7178	0.3871	0.1891	0 2361
Cell volume W_{cell}	1 2331	1 1745	0.8788	0 8083

Samples were suspended in 10 mM imidazol buffer plus NaCl in th concentration shown. Procedures as described under Materials and Methods.

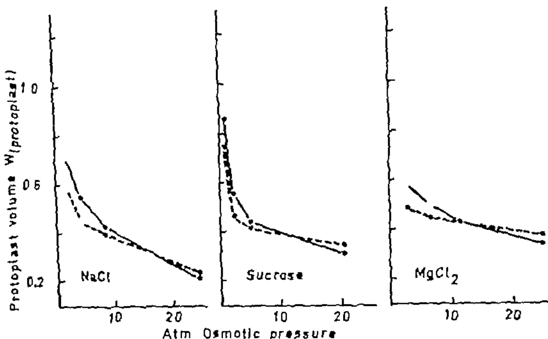


Fig. 1 Sucrose exclusion space (protoplast volume) in *N meningitidis* competence variants. Swelling and shrinkage due to variation in the osmotic pressure. Solid lines *cp* variant dotted lines *cp* variant.

from *cp* ones. The direct measurements from each experiment were statistically analysed. The significance of the difference between the means was tested in the Student *t*-test. Analysis of variance was also performed in several experiments (3)

RESULTS

Volume measurements in intact cells. The effect of changes of medium osmotic pressure on cell size (dextran space) and protoplast size (sucrose space) was measured in the *N meningitidis* competence variants. Fig. 1 shows that an increase of the osmotic pressure by NaCl, sucrose or $MgCl_2$ above the physiological pressure of around 7–8 atmospheres caused a pronounced shrinkage in protoplast volume whereas a reduction in osmotic pressure resulted in an even more pronounced swelling. It is seen that the decrease in volume with equosmolal concentrations seems somewhat more pronounced if NaCl is the solute than if sucrose or $MgCl_2$ are used. Fig. 2 shows that the salts caused an additional, marked shrinkage in the volume of the whole cell, whereas the non electrolytic sucrose had hardly any effect on the cell volume.

The experiments demonstrated a significant difference between the competence variants. The protoplasts from the cp^+ variants can obviously swell to a much larger size, and shrink to a somewhat smaller size than those from the cp^- variant. At 0.05 M sucrose the protoplast size of the cp^- cell was averagely 88 per cent of that of the cp^+ one (6 experiments). At 0.7 M sucrose the cp^- protoplast size was averagely 109 per cent of the cp^+ protoplast size (6 experiments). The swelling and shrinkage of the cell volume as measured in the presence of NaCl or MgCl were also more pronounced in the cp^+ variant (Fig. 2).

Comparison of spheroplast size. The size of spheroplasts obtained by autolysis and by the penicillin technique was next compared in the competence variants as described under Materials and Methods. In several experiments with autoplasts, those from cp variants were significantly smaller than those

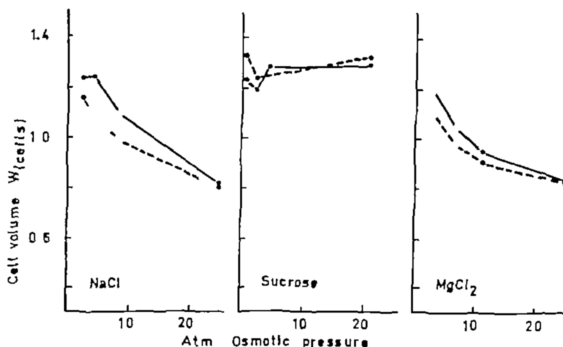


Fig 2 Dextran exclusion space (cell size) in *N meningitidis* competence variants. Swelling and shrinkage due to variation in the osmotic pressure. Solid lines: *cp* variant dotted lines *cp⁻* variant.

from cp^+ ones $(0.01>p>0.001)$ The relative size (diameter) of the spheroplasts is recorded in Table 2. The average diameter of the penicillin spheroplast was a little larger than that of the autoplasts under otherwise identical conditions. Also, the populations of penicillin spheroplasts were more heterogeneous with regard to size. The statistical significance of the difference in average size of penicillin spheroplasts from the competence variants was also less marked. Out of four experiments, two resulted in $0.02<p<0.01$ whereas two gave $0.01<p<0.001$

DISCUSSION

The measurements by the exclusion space technique using sucrose and dextran corroborate the findings previously obtained in the competence variants which were based on absorbancy measurements (4) The variation in the volume of the protoplast during changes in osmotic pressure follows the change in absorbancy This was indeed to be expected since the turbidity changes are considered an effective measure of the volume of the protoplast (1 4) Both experimental methods indicate strongly that the protoplasts

TABLE 2. *Relative Size of Spheroplasts from* N meningitidis *Competence Variants*

Type of spheroplasts compared	Relative diameter		Significance of the difference
	p	*cp*	
Penicillin spheroplasts	100 %	89 6 %	$0.01<p<0.001$
Autoplasts	100 %	90 1 %	$0.01<p<0.001$

Measurements and calculation of significance as described under Materials and Methods. The relative size is the average of two experiments

from the competence variants are different. The capacity for swelling and shrinkage is obviously far more pronounced in the *cp* protoplast.

If the protoplasts are suspended in NaCl they may apparently shrink to a size smaller than that to be seen after suspension in sucrose or $MgCl_2$. It has previously been found that NaCl in a high concentration counteracts the induction of cell-wall and membrane lysis which is usually connected with conditions conductive to plasmolysis (5 6) Thus, it cannot be precluded that the protoplast shrinkage in the presence of sucrose or $MgCl_2$ is limited by a lysis of the membranes (osmotic barrier) a lysis which is less pronounced in the NaCl assay

Increasing salt concentration causing decreases in the volume of the protoplast also brings about contraction of the cell wall. This shows that the cell wall of *N. meningitidis* like that of *E coli* is not completely rigid (1 7) As in *E coli* salts (NaCl or MgCl) are required if significant contraction is to be provoked, whereas the change in wall space along with increasing concentrations of sucrose is insignificant. The decrease in whole cell volume has been ascribed to some interaction with the murein (8) or to an increased pull caused by adherence points between the wall and cytoplasmic membrane accordingly as the protoplast decreases in size (1) It has been suggested that the maintenance of the adherence points between the wall and the membrane in *E. coli* depends on the ionic strength of the plasmolysing agent (1) If the same applies to *N meningitidis* this could mean that rupture of the adhesion points during plasmolysis is a requirement for induction of autolysis of cell wall and cell membrane (5 6)

The cell-space measurements indicate that the *p* protoplast can swell to a larger size and shrink to a somewhat smaller than the *p* protoplast. But at physiological osmolality around 7 8 atmospheres, the protoplast volumes of the competence variants are not very different. This could possibly mean that the *p* protoplast is in fact larger but at physiological osmolality is provided with cytoplasmic invaginations or even true mesosomes that are collapsed (4) This hypothesis is also of interest with a view to the role assigned to mesosomes in transformation (10 11 12)

In fact, comparison of spheroplast size indicates that cp^+ autoplasts are significantly larger than *cp* autoplasts. The difference between cp^+ and *cp* penicillin spheroplasts is less significant. But penicillin spheroplasts enlarge during growth in the "spheroplast broth (6) and become very heterogeneous with regard to size. It is likely that the autoplasts rather are representative of the average protoplast size of the actively growing cell population as measured by the exclusion space technique.

It is a great pleasure to acknowledge the expert technical assistance of Miss *Lydia Gikle*

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EFFECTS OF EXPOSURE TO HIGH AND LOW OSMOTIC PRESSURE UPON *NEISSERIA MENINGITIDIS* TRANSFORMATION

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Jyssum, K. Effects of exposure to high and low osmotic pressure upon *Neisseria meningitidis* transformation. Acta path. microbiol. scand. Sect. B, 84: 45–50, 1976.

Transformation efficiency in competent variants of the *Neisseria meningitidis* Strain M1 increased more than three-fold after exposure of the recipient cells to approximately 35 atmospheres osmotic pressure for 20 min. Growth of the recipient cells at increased osmotic pressure constantly reduced the transformation efficiency. Exposure of the cells to hypotonic conditions reduced transformability significantly and attempts to restore transformability by means of supernatant solutions from competent cultures were unsuccessful. Incompetent variants of the Strain M1 could not be rendered competent by exposure to increased tonicity. The findings have been discussed in relation to the effects of tonicity variation on *N. meningitidis* competence variants.

Key words: *Neisseria meningitidis*; transformation; osmotic pressure.

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The influence of variation in tonicity on transformation has not been extensively studied, but two apparently different effects have been described. In a few bacteria such as *Bacillus licheniformis* (20, 21) and *Bacillus amyloliquefaciens* (1), transformation is significantly enhanced by high osmolarity (up to 0.6–0.8 M), whereas it appears that most of the transformable bacteria do not benefit from increased salt concentrations. But it is possible that transfection in some instances is increased if occurring in slightly hypertonic solutions (19).

The other effect of osmotic change has been observed in *Bacillus subtilis* (17). In this case, osmotic shocking of competent bacteria reduced transformability by 80–90 per cent by way of a depression of the uptake of DNA. The supernatant solutions from competent bacteria restored transformation and DNA uptake. This observation was discussed in relation to the finding that many surface-located enzymes and binding proteins are released from *Escherichia coli* and other micro-organisms by cold osmotic shock (3, 16). It was suggested that the shock in *B. subtilis* released a competence factor of protein nature from the surface of the bacteria.

The reactions of competent and incompe-

tent variants of the *Neisseria meningitidis* Strain M1 to variations in the osmotic pressure have previously been examined (9 10 11) Several observations indicate that the membranes of the competent variants differ from those of the incompetent ones. The findings have been interpreted to the effect that the cytoplasmic membranes of the competent variants may shrink more freely upon extrusion of water and swell upon uptake of water without deleterious effects.

The present work was undertaken to find out whether the shrinkage and swelling to be observed after variations in the osmotic pressure (9) could be correlated with the capacity of the cells to be transformed.

MATERIALS AND METHODS

Bacteria. The wild type *N meningitidis* Strain M1 of serogroup B was used along with the auxotrophic mutants M1-6 *his pro* and M1-8 *his arg* Variants which were competent in transformation were indicated by the symbol *p* and incompetent ones by *p* The growth requirements as well as competence in transformation were controlled as described previously (5 6)

Media and growth. Blood agar plates and Heart Infusion Broth (HIB Difco) agar plates were used as solid complete media. HIB served as complete fluid medium. The basal media were those used before (4) Growth was performed and followed by measuring absorbancy or colony-forming units (CFU) as previously described (8)

Preparation f DNA Transforming DNA was prepared according to a modification of the Marmur procedure (14) Streptomycin resistant DNA was isolated from a single-step, high level resistant mutant (5)

Exposur of recipient cells to tonicity variation. The recipient cells were generally prepared from cultures in HIB. Batch cultures (10 or 12 ml) were inoculated from a start culture in the exponential phase to an absorbancy of approximately A = 0.100 and grown during shaking t 37 C. The absorbancy was followed and, at the desired growth phase, the cells were harvested in the centrifuge (2500 × g for 10 min) and resuspended in the salts of the basal medium (2, 4) Growth at increased *osmotic pressure* took place after resuspension of a log phase culture in HIB supplemented with 0 7 M sucrose and 0.05 M $MgCl$ (10, 11) Samples f the recipient cell suspensions were exposed to hypertonic and hypotonic conditions, usually for 20 min. The technique followed the same lines as those in previous studies of tonicity variation in *N meningitidis* (9 10, 11)

Transformation The influence of tonicity variation was tested in a transformation system containing 1 7 ml HIB with 0.005 M $CaCl_2$ 0.2 ml receptor cell suspension 0.1 ml transforming DNA in NaCl-citrate buffer (0.15 M NaCl + 0.015 M Na_3-citrate, pH 7 4) The receptor cell suspension was diluted before use to ensure that it contained approximately 10^8 CFU per ml (14) Transformation took place at 37 C and was terminated after the desired time usually 45 min, by the addition of 0 1 ml deoxyribonuclease (DNase) giving a final concentration of 50 μg/ml (12, 14)

RESULTS

Transformation after exposure to increased tonicity Samples of the competent *N meningitidis* cells were harvested at various times during the growth of batch cultures, exposed to increased tonicity (approximately 32 atmospheres osmotic pressure) and subsequently transformed in the *str* locus. Fig 1 shows that the transformation frequency was enhanced throughout the phase of exponential growth, and nearly to the same extent after treatment had lasted for 20 min. It is seen that the exposure also resulted in an increased number of transformants per ml in spite of the lethal effect (9) It is also noted that the recipient cells were more susceptible to the killing effect of osmotic stress during the very early log phase than at later stages of the growth cycle. It has previously been observed that stationary phase cells are less affected by tonicity variation than those from the exponential phase (10)

Fig 2 shows that the effect on transformation was also dependent upon the tonicity Maximal effect was obtained at approximately 35 atmospheres osmotic pressure if the cells were exposed for 20 min. But the enhancement in transformability is apparently not dependent upon the type of solute used since the results obtained virtually were identical whether sucrose or NaCl were used.

The results obtained by subsequent transformation of the markers *his arg* and *pro* were similar to those reported to be obtained in the case of *str* marker

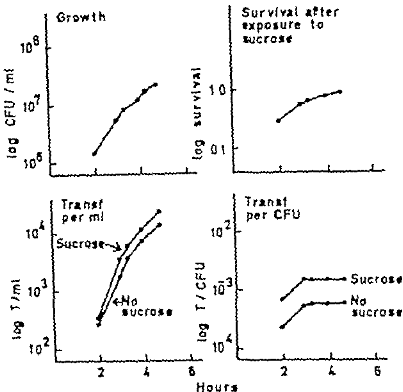


Fig 1 Influence of exposure to increased tonicity on the transformation in N. meningitidis. Relation to the growth cycle. Ten ml batch cultures were grown during shaking at 37 C. At the times indicated, the cells were harvested, exposed to 30 per cent sucrose for 20 min in the presence of the salts of the basal medium (2, 4) and subsequently transformed to streptomycin resistance. Technique as described under Materials and Methods. CFU = colony-forming units.

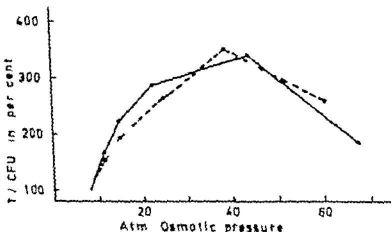


Fig 2 Influence of exposure to increased tonicity on the transformation in N. meningitidis. Relation to the osmotic pressure used. Batch cultures were grown during shaking to the exponential phase (approximately A = 0.330). The cells were harvested and exposed for 20 min to the salts of the basal medium (2, 4) plus sucrose or NaCl at the osmolality shown. The cells were subsequently transformed to streptomycin resistance. Technique as described under Materials and Methods. Solid lines exposed to NaCl. Dotted lines exposed to sucrose.

TABLE 1 *Influence of Growth at Increased Tonicity on the Transformation Frequency in N meningitidis*

Growth medium	Approximate osmotic pressure (Atmospheres)	Transformation	
		T/CFU	Per cent
HIB	8	1.54×10^{-3}	100
HIB + sucrose 5 per cent	12	$2.4[illegible] \times 10^{-[illegible]}$	15.7
HIB + sucrose 10 per cent	15	$1.61 \times 10^{-[illegible]}$	10.4
HIB + sucrose 20 per cent	25	1.25×10^{-4}	8.1

Batch cultures in HIB supplemented with 0.05 M $MgCl_2$ and sucrose as shown were inoculated from a HIB culture in the exponential phase and grown to A close to 0.300. Transformation to streptomycin resistance as described under Materials and Methods. T/CFU = transformants per exposed colony-forming unit.

Transformation after growth at increased tonicity Growth of *N meningitidis* can take place at increased tonicity after a period of adaption (10 11) Under these conditions, however the growth rate is considerably slowed down particularly in competent strains. Table 1 shows that growth at increased osmotic pressure resulted in a pronounced reduction in transformation frequency The reduction was significant already after a moderate increase in tonicity (5 per cent sucrose)

If cells growing under increased osmotic pressure were re-inoculated in complete medium (HIB at approximately 8 atmospheres osmotic pressure) and growth had been resumed, the transformation frequency returned to that characteristic of this medium.

Transformation after exposure to reduced tonicity Cells from *N meningitidis* that are exposed to hypotonic conditions are osmotically shocked (9) Such treatment regularly resulted in a reduction of the transformation efficiency as shown in Table 2. This is in agreement with the findings in studies of transformation in *B. subtilis* (17) But, unlike the situation in the latter transformation system, attempts to restore the transformability by means of supernatant solutions or extracts from competent cells were unsuccessful. Numerous experiments using supernatants prepared by osmotic shocking (17) and autolysis (10) as well as crude cell extracts in various buffers and in saline were performed, but they were altogether negative

If osmotically shocked *N meningitidis* cells were inoculated into complete medium, transformation efficiency was restored after a lag

TABLE 2. *Influence of Exposure to Decreased Tonicity on the Transformation Frequency in N meningitidis*

Treatment of the recipient cells	Addition of supernatant	Transformation	
		T/CFU	Per cent
None	—	1.65×10^{-8}	100
None	+	1.70×10^{-8}	103
Exposed to distilled water	—	$3.63 \times 10^{-[illegible]}$	22
Exposed to distilled water	+	$4.46 \times 10^{[illegible]}$	27
Exposed to $MgCl_2$ 0.005 M	—	$8.92 \times 10^{-[illegible]}$	54
Exposed to $MgCl_2$ 0.005 M	+	$1.03 \times 10^{[illegible]}$	63

Competent *N meningitidis* cells were exposed to hypotonic conditions for 20 min, incubated for 30 min with (+) and without (—) "shock fluid" from a competent strain and transformed to streptomycin resistance. Technique as described under Materials and Methods. T/CFU = transformants per exposed colony forming unit.

period at the approximate time when the growth was resumed to that typical of growth in HIB.

Attempts to induce competence in incompetent variants by exposure to increased tonicity. Numerous attempts were made to induce competence of transformation in *cp⁻* variants of the *N. meningitidis* Strain M1 by exposure of the cells to hypertonic conditions. Transformation of the following markers *str, his, arg* and *pro* was attempted. But a number of transformants significantly higher than that observed in the control experiments in which mutation was assessed was not seen in any of the experiments.

DISCUSSION

These experiments demonstrate that the transformation efficiency is significantly enhanced by transitory exposure of competent *N. meningitidis* cells to increased tonicity. It should be emphasized that prolonged exposure of the cells to increased osmotic pressure always resulted in a very pronounced reduction in transformability and the same applies to attempts at performing the transformation in a system with high osmolality (*Jyssum* unpublished results).

The recipient cells are definitely damaged by high osmolality and, since it is known that *cp* cells are more easily damaged than *cp⁺* cells (9, 10, 11), it is suggested that the effect may be due to a preferential killing of any *cp* cells that might be present in the population (7, 13). But the finding that even the overall number of transformants per ml is increased subsequent to the exposure argues in favour of a true increase in transformation.

Several events follow the exposure of *N. meningitidis* cells to increased tonicity. The first effect is a contraction of the cytoplasm (9) which when it is extensive, results in plasmolysis (10). Plasmolysis obviously damages the cell. An early effect of this damage is a reduced capacity of the cell membrane to maintain an osmotic barrier due to increased permeability (9). This might also involve increased permeability of transforming DNA. In this connection it is of importance that the early damage caused by plasmolysis can be repaired by subsequent incubation in a medium such as the one used in transformation (9).

Plasmolysis also triggers a successive autolysis of the cell wall as well as of the cell membrane (10, 11). Either one of these phenomena could be of importance. It has previously been found that autolysis is of minor degree, even absent after the exposure to 0.5–0.7 M NaCl, whereas it certainly takes place after similar exposure to high concentrations of sucrose (10). Since exposure to high concentrations of sucrose and of NaCl increases the transformation to almost the same extent, it seems as if autolysis may play no major part in this enhancement.

These findings should be considered in relation to the role assigned to mesosomes and cytoplasmic membranes in transformation (22, 23, 24). *Ryter* (18) proposed that mesosomes and cytoplasmic invaginations which interdigitate the cytoplasm unfold under hyperosmotic conditions and produce the gaps between the cytoplasm and the cell wall. She also suggested that each nucleus is connected with the cell envelope by one of the mesosomes. Thus exposure to high tonicity may alter the communication between the mesosomes or cytoplasmic invaginations and the external milieu of the cell, thus favouring transformation. Experiments reported before (2) show that the exposure of recipient cells to increased osmotic pressure also results in an enhancement of the DNA uptake.

The increase in transformation efficiency is associated with exposure to increased tonicity. In cultures growing under increased tonicity the transformation efficiency is reduced. But in such cultures the growth rate is slowed down, indicating a decrease in the overall metabolism (10). Thus, the observation agrees with the previous finding that the transformation efficiency in *N. meningitidis* is directly related to the metabolic activity of the recipient cell population (15).

Competent *N meningitidis* cells may be rendered less efficient in transformation by exposure to conditions generally conductive to plasmoptysis (9) Since supernatant fractions could not restore transformability in the osmotically shocked cells, the situation is probably different from that described in reports on *B. subtilis* (17) Accordingly the findings do not lend support to the hypothesis according to which a competence factor in *N meningitidis* is released from the surface of the bacteria by osmotic shocking It has previously been shown that the membranes in *N meningitidis* cells are damaged by osmotic shocking (9 11) and it seems likely that the reduction in transformation efficiency is due to an impairment of the membrane integrity

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SURVIVAL OF ANAEROBIC BACTERIA DURING TRANSPORTATION

I Experimental Investigations on the Effect of Evacuation of Atmospheric Air by Flushing with Carbon Dioxide and Nitrogen

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Justesen, T & Lykkegaard Nielsen, M. Survival of anaerobic bacteria during transportation. I Experimental investigations on the effect of evacuation of atmospheric air by flushing with carbon dioxide and nitrogen. Acta path. microbiol scand. Sect. B, 84 51–56 1976.

The effect of evacuation of atmospheric air during transportation on recovery of anaerobic bacteria was investigated. Evacuation of atmospheric air from glass tubes by flushing with pure carbon dioxide lowered the content of oxygen to about 0.4 per cent. Three *B. fragilis* strains and one strain of *Fusobacterium mortiferum* and of *Peptostreptococcus anaerobius* were investigated. Bacterial recovery was determined one hour and 24 hours after evacuation of atmospheric air by pure carbon dioxide and pure nitrogen, and was compared to bacterial recovery from samples transported with free access to atmospheric air. Evacuation by pure carbon dioxide significantly improved the recovery of one *B. fragilis* strain after 24 hours of transportation and significantly impaired the recovery of *P. peptostreptococcus anaerobius* after one hour of transportation, while evacuation by pure nitrogen significantly improved the recovery of *P. peptostreptococcus anaerobius* after 24 hours of transportation. In all other cases, however no statistically significant effect on bacterial recovery was found

Key words Anaerobic bacteria transportation.

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Transportation of clinical samples for anaerobic culture is a problem, because of the injurious effect of the oxygen in the atmosphere on anaerobic organisms, the varying sensitivity of different anaerobic bacterial strains to oxygen being familiar As a standard procedure however evacuation of atmospheric air from tubes containing samples for anaerobic culture by flushing with carbon dioxide or nitrogen has been recommended (2, 9).

We have used the method in question in our work with clinical and experimental anaerobic infections (5 6 7) To our knowledge, however the significance of the flushing procedure has never been investigated on a quantitative scale. The aim of the present study was to investigate the effect of evacuation of atmospheric air on survival of anaerobic bacteria, by flushing with carbon dioxide and nitrogen.

MATERIAL AND METHODS

Bacterial Test Strains

The test strains included two different strains of *B. fragilis* ss. fragilis from human abscesses in pure culture (Lab. nos. IMM 3362 and IMM 21328);

one *B. fragilis* strain isolated from a case of human biliary tract infection and not fitting into any known subspecies (Lab. no IMM 2183) one stock culture of *Fusobacterium mortiferum* (VPI 7450) and one of *Peptostreptococcus anaerobius* (VPI 5459) Four of the five bacterial strains have previously been used in experimental anaerobic infections (6)

Anaerobic Culture Procedures

The basic principles of anaerobic culture, employing a glove-box and pre-reduced, anaerobically sterilized media, have already been described (3) Cultures of test strains were grown in Peptone-Yeast Extract basal medium (PY) and colony counts were made on Brain Heart Infusion Agar (Difco) supplemented by defibrinated horse blood 5 per cent, yeast extract, cysteine, haemin and menadione (BAP medium) BAP media were stored for t least 24 hours in the glove-box prior to use. Inoculated media were incubated in the glove-box at 35 C in an atmosphere of 80 per cent nitrogen, 15 per cent hydrogen and 5 per cent carbon dioxide.

Flushing of Samples with CO and N

In th glov -box, ten-fold dilutions of 24-hour cultures in PY medium were mad in T 2 buffer solution (NaCl 4 g K_2SO_4, 5 g KH_2PO_4, 1.5 g Na_2HPO_4, 3 g $MgSO_4$, 0.12 g $CaCl_2$, 0.11 g and gelatine, 0.01 g/l distilled water) (8) One ml of a suitable dilution was mixed in 100 ml T buffer aiming at a bacterial concentration of 10^2–10 bacteria per ml. The bacterial concentration of this suspension (from now on and in the tables referred to as initial bacterial suspension) was determined by counting 8–10 BAP plates each flooted with ¼ ml suspension. Ten glass tubes, each containing 3 ml of the initial suspension, were taken outside the glove-box and shaken for two minutes in atmospheric air From each of the 10 tubes, 1.5 ml was transferred to each of two other glass tubes, one of which was left with free access to tmospheric air while the other was flushed with oxygen-free CO for 60 seconds (9 1 per minute) After one hour vital counts were made from all t bes after they had been returned to the glove-box (10 tubes flushed with CO_2 and 10 tubes left in tmospheric ai) by floating BAP plates in duplicate with ¼ ml. Corresponding experiments were carried out after 4 hours instead of one hour and corresponding experiments were also carried out after flushing with N instead of flushing with CO_2.

The experiments were so arranged that flushing of samples was performed in a room next to the surgical ward and all samples were actually transported from the surgical ward to the bacteriological laboratory imitating the transport of ordinary clinical samples.

Determination of Residual Oxygen after Flushing with CO_2

The oxygen tension was measured in 30 glass tubes flushed w th oxygen-free CO (9 1 per minute) for 60 seconds and stoppered with rubber stoppers. The analyses were carried out on the Digital Acid-Base Analyser PHM 72 (Radiometer) Before every analysis, the apparatus was calibrated both to the partial pressure of oxygen in the atmospheric air and to zero by using PO_2 Zero Solution (Radiometer type 10–S 4150)

Determi ation of pH after Flushing with CO

Determinations of pH were carried out in 20 samples of rabbit serum and in 15 samples of T 2. Sample sizes, glass tubes and flushing procedures were similar to those used in the experiments on bacterial recovery Analyses were carried out on a pH-meter (pH-meter 28, electrode GK 2321C, Radiometer) and the apparatus was calibrated to pH 6 50 before every analysis (Pufferlösung S 1001 Radiometer) Determinations of pH were made before and one hour after flushing with CO_2.

Statistical Calculations

Analysis of the ariance of bacterial counts (x) within each experiment based on 10 trials showed that the ariance could be assumed to be directly proportional to the mean value ($s^2 = k\bar{x}$) Bacterial strains no. 1–4 had factor of proportionality in common in all experiments with the same bacterial strain, and k could be assumed to be the same value, with k = 2.892. In the case of bacterial strain no. 5 k = 1 388 was found, apart from the 24-hour values in Table . The t test and the calculation on the 95 per cent conf dence limits were determined from the mean of the logarithm of the bacterial counts ($y = \log(x)$) as $\bar{y} = \overline{\log(x)}$ within each experiment based on 10 trials is assumed to be normally distributed, with parameters ($p, \frac{k}{t\ n}$) p = 9 t = 2 and n = 10

Changes in pH of rabbit serum and T 2 solution was nalysed by the Mann-Whitney test

RESULTS

Table 1 summarizes the bacterial recovery from samples flushed with carbon dioxide and from samples left with free access to atmospheric air for one and 24 hours after

TABLE 1 *Evacuation of Atmospheric Ai by Pure Carbon Dioxid from Samples During Transportation—the Effect on Recovery of Five A aerobic Bacterial Strains.*

Bacterial test strain	In tial bacterial suspension. Bacteria/ml and SEM *	Bacterial recovery after 1 h		Initial bacterial suspension. Bacteria/ml and SEM	Bacterial recovery after 24 h.	
		Samples flushed with CO	Samples in atmospheric air		Samples flushed w th CO_2	Samples in atmospheric air
B. fragilis	121	97 1 %	102.1 %	121	62.9 %	17.9 %
ss. fragilis	5.9	(70.9–132.9)	(74.9–139.3)	5.9	(44.2–89 7)	(10.8–29 7)
B. fragilis	467	89 1 %	91.0 %	467	68.1 %	67.3 %
ss. fragilis	11.6	(75 7–105 0)	(77.3–107 1)	11.6	(57 1–81.2)	(56.4–80.3)
B fragilis	794	93.8 %	90.. %	794	74 1 %	75 7 %
ss. no fit	15..	(82.8–106 1)	(79 6–102.2)	15.2	(65.0–84.6)	(66 4–86.3)
Fusobact. mortiferum	897	82.6 %	81 7 %	171	60 1 %	69.3 %
	16 1	(73.2–93.2)	(72.4 92.1)	7.0	(44 5–81.3)	(51.8–92.8)
. Peptostrep.	42	67.6 %	89.3 %	100	0	0
anaerobius	5.8	(57.0–80.1)	(76.3–104.5)	3 7		

SEM standard error of the mean
Each alue given n per cent is the mean alue from 10 trials with 95 per cent confidence limits given in brackets

the flushing procedure In the three *B. fragilis* strains tested, a small but statistically insignif icant reduction in bacterial recovery is found after one hour but no significant difference between samples flushed with carbon dioxide and samples left in atmospheric air In *Fusobacterium mortiferum* a statistically significant reduction in bacterial recovery of about 20 per cent is found after one hour but no significant difference between samples flushed with carbon dioxide and samples left in atmospheric air In *Peptostreptococcus an*

TABLE 2 *Evacuation of Atmo pheric Air by pure Nit ogen f om Samples during Transportation—the Effect on R covery of Five Anaerobic Bacterial Strains.*

Bacterial test strain	Initial bacterial suspension. Bacteria/ml and SEM	Bacterial recovery after 1 h.		Initial bacterial suspension. Bacteria/ml and SEM	Bacterial recovery after 24 h.	
		Samples flushed with N	Samples in atmospheric air		Samples flushed with N	Samples in atmospher air
1 B fragilis	968	88 5 %	92.3 %	968	10.2 %	11.6 %
ss fragilis	16 7	(78 8–99 0)	(82.4–103.3)	16 7	(7.9–13.2)	(9.2 14.6)
2 B fragilis	295	90.2 %	92 0 %	295	77 4 %	75 7 %
ss fragilis	9 2	(73.4–110.7)	(75 1 112.9)	9.2	(62.5–95.9)	(61.0–93.9
3 B fragilis	161	104 7 %	93.8 %	161	94.6 %	92.5 %
no fit	6 8	(80 2 136.8)	(71..–123 4)	6.8	(71 9–1 4.5)	(70.2–121
4 Fusobact	101	110 7 %	105 0 %	477	70.8 %	77.3 %
mortiferum	3 4	(79 3–154.5)	(74.9–147 1)	11 7	(59 6–84.0)	(65.5–91 4
5 Peptostrep	160	76 6 %	81 1 %	462	21.9 %	1 4 %
anaerobius	4 7	(62 7 93 7)	(68.5–98.9)	8.0	(8.2 38.6)	(0.2–12.9)

SEM standard error of the mean.
Each alue given in per cent is the mean alue from 10 trials with 95 per cent confidence limits given in brackets.

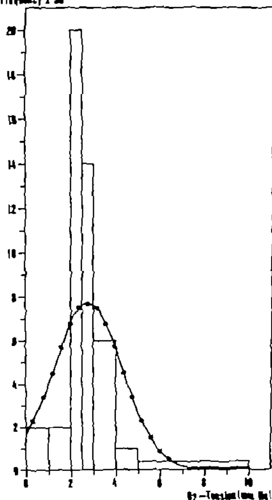


Fig 1 Oxygen-tensions in glass tubes after evacuation of atmospheric air by flushing with carbon dioxide.
Columns Observed oxygen tensions.
●—●—●—● Density of the normal distribution with the estimated parametres (mean 2.8, variance 2.5)

aerobius a 32 per cent significant reduction in bacterial recovery was found in samples flushed with carbon dioxide after one hour while a reduction of 11 per cent was found in samples left in atmospheric air The difference between the two groups is significant ($p < 0.01$)

In the three *B fragilis* strains tested, a varying but in all three strains significant reduction in bacterial recovery is found after 24 hours in samples flushed *with carbon* dioxide. Only in *B fragilis I* however the reduction in bacterial recovery is significantly different in samples flushed with carbon dioxide (37 per cent reduction) and samples left in atmospheric air (82 per cent reduction) ($p < 0.001$) In *Fusobacterium mortiferum* and in *Peptostreptococcus anaerobius*, a significant reduction in bacterial recovery did not reveal any significant differences between samples flushed with carbon dioxide and samples left in atmospheric air after 24 hours.

In Table 2, bacterial recovery one and 24 hours after flushing with nitrogen is summarized. In all five bacterial strains tested after one hour no significant differences between samples flushed with nitrogen and samples left with free access to atmospheric air were found. Only in *Peptostreptococcus anaerobius* a significant reduction in bacterial recovery was found in both groups (about 20 per cent) but without any significant intergroup difference.

In all five strains tested except *B fragilis III* a significant reduction in bacterial recovery is found after 24 hours compared to

TABLE 3 *Oxygen-tension after Evacuation of Atmospheric Air from Glass Tubes by Flushing with Carbon Dioxide*

Number of trials	Mean	Range	O_2-tension after flushing (mmHg) Percentiles 5	10	50	90	95
30	2.8	0.6–9.1	0.7	1.4	2.6	5.4	5.1

2.8 mmHg = 0.39 % O_2.

TABLE 4 *Changes in pH in Rabbit Serum and in T-2 Solution after Flushing 1.5 ml Samples with Carbon Dioxide (Median and Range)*

	Number of trials	pH Before flushing	pH After flushing
Rabbit serum	20	7.48 (7.46–7.50)	6.38 (6.30–6.42)
T 2*	15	7.00 (6.98–7.02)	6.00 (5.94–6.02)

cf. text.

the initial bacterial suspension. The only significant difference between samples flushed with nitrogen and samples left in atmospheric air however was found in *Peptostreptococcus anaerobius* ($p < 0.05$) after 24 hours.

Figure 1 and Table 3 show the effect of flushing with carbon dioxide on evacuation of oxygen from glass tubes. It is seen from Figure 1 that the distribution was not normal but was skewed to the right. The median per cent oxygen left after evacuation was 0.37 per cent (95 per cent confidence limits 0.31–0.41). The limits of the range of oxygen tensions found (0.6 and 9.1) are equivalent to 0.08 and 1.3 per cent oxygen.

Changes in pH of rabbit serum and of T 2 solution appear in Table 4. A significant fall in pH was found both in serum and T 2 solution, and a small, but significant difference between the change in pH in the two types of samples investigated ($p < 0.001$).

DISCUSSION

It is generally accepted that CO_2 in low concentrations stimulates the growth of anaerobic bacteria to varying degrees. It has been shown, however that pure CO_2 almost completely suppresses the growth of *Clostridium perfringens* from spores (1) and that relatively pure CO_2 maybe inhibits the growth of some Gram-negative anaerobic bacteria (11). In our experimental model, an inhibitory or killing effect of pure CO_2 was observed in the case of *Peptostreptococcus anaerobius* within the transportation time of one hour whereas the reverse effect of CO_2 was observed in *B. fragilis I* within the transportation time of 24 hours. The more precise nature of the effect of pure CO_2 on bacteria in a stationary phase in a non-growth-supporting solution has not been elucidated in this preliminary investigation. Since however pure CO_2 can display a reverse effect on two different anaerobic strains, further investigations, including a greater number of strains, should be carried out under conditions more similar to those encountered in clinical bacteriology before evacuation of atmospheric air with pure CO_2 is recommended for transportation of samples for anaerobic culture. In this study very low bacterial concentrations were deliberately chosen in order to avoid uncontrollable bacterial interactions and to avoid carry-over of nutritive factors from the growth medium.

It appears from Table 1 and Table 2 that, if transportation time is kept below one hour evacuation of atmospheric air is of minor importance, since there was a reduction in bacterial recovery of only 20 per cent among the most sensitive strains tested. As regards 24 hours transportation, evacuation of atmospheric air with pure CO_2 had no effect on bacterial recovery in strains number 2, 3, 4 and 5 and evacuation with pure nitrogen had no effect on bacterial recovery in strains number 1, 2, 3 and 4. Whether this proportion of strains not benefitting from the removal of atmospheric air holds true when a greater number of strains is examined, remains to be investigated, and cannot be predicted from the present study.

In a recent work by *Tally et al.* (10) 57 freshly isolated anaerobic strains were tested for oxygen tolerance (survival after exposure to atmospheric oxygen). Fifty three of the 57 strains survived exposure to atmospheric air for 24 hours or more while the remaining four strains tolerated at least eight hours of exposure. Ten *B. fragilis* strains tested, all survived 48 hours or more; this observation parallels the results obtained in our investigation (cf. Table 1 and 2) although a closer

comparison is not possible since *Tally et al* in the case of the single bacterial strain did not quantitate the decline in bacterial survival after exposure to atmospheric oxygen.

In more than 95 per cent of the trials summarized in Table 3 less than one per cent oxygen was left in the tubes after flushing with carbon dioxide *Loesche* (4) investigated the oxygen sensitivity of a number of growing anaerobic bacterial strains. He showed that strains of anaerobic bacteria often encountered in clinical specimens (*B. ovalis B melaninogenicus B fragilis F nucleatum* and *Cl. novyi* type A) survived oxygen tensions of one per cent or more The flushing procedure used in this study should therefore be sufficient for the evacuation of oxygen. The median residual oxygen of 0.37 per cent is probably in part due to the handling of the stoppers. The stoppers must be handled carefully in order to prevent oxygen from slipping in. The skewed distribution shown in Figure 1 is probably due to the fact that most of the uncontrollable small deviations in the flushing procedure can only increase the amount of oxygen

The buffer capacity of T 2 solution is due to the content of primary and secondary phosphates. Flushing with carbon dioxide lowered the pH from 7 0 to 6 0 As all the test strains used in this study have been cultured in Peptone Yeast extract medium with a final pH in the range of 6.0–6.5 for several days without any loss of viability it is unlikely that this change in pH could explain any killing effect of CO (Table 1 strain number 5)

From this preliminary study we conclude that a transportation time of less than one hour ensures a good recovery of even the rather sensitive strain of *Peptostreptococcus anaerobius* We further conclude that the extent to which flushing of samples with carbon dioxide or nitrogen improves the recovery of anaerobic bacteria is uncertain and should be investigated on a larger scale before the method is generally recommended.

Our thanks are due to *Susanne Moller* Biostatistical Department, Statens Seruminstitut, who kindly adviced us on the statistical evaluation. The skilful technical assistance of *Ed I Hoen* and *Anette Damstor-Andersen* is gratefully acknowledged.

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MULTIPLICATION OF *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS* IN *MICROTUS AGRESTIS* (FIELD VOLE)

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Jespersen, A. Multiplication of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in *Microtus agrestis* (field vole) Acta path. microbiol. scand. Sect. B 84 57-60 1976

The multiplication of bacteria is examined by quantitative culture from the organs of tw groups of field voles injected intraperitoneally w th a large dose of *M tuberculosis* or a small dose of *M bovis* and killed at intervals during period of p t 3 months after the injection. *M tuberculosis* given in a dose of 7 × 10⁷ viable units did not multiply or multiplied only to small extent. None of the animals in the group died from tuberculosis and the macroscopical lesions found at autopsy were insignificant. In contrast, a dose of 14 viable units of *M bovis* provoked generalized tuberculosis running a rapid, fatal course. The bacteria multiplied almost uninhibited throughout the course of the infection.

Key words *Mycobacterium tuberculosis Mycobacterium bovis* multiplication *Microt s agrestis.*

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Experiments by *Wells* (1938) and *Griffith* (1939) indicated that the organism of *Microtus agrestis* is a poor environment for the multiplication of *M tuberculosis* but ex tremely favourable for *M bovis* In the pre sent study the multiplication of the two spe cies has been examined by quantitative culture from the organs of infected animals.

MATERIAL AND METHODS

Experimental The experiment comprised 1 cap tured voles Ten animals were injected intraperitoneally with 10 mg of culture of a irulent strain of *M tuberculosis* (E 1068311) and leven with 10⁻⁸ mg of a virulent strain of *M bovis* (T 5605B) One animal from each group was killed at various i tervals d ring the period from the 6th day up to 2-3 months after the injection, and the number of viable unit in the organs wa determined by quantitative culture.

I f ction The bacteria were grown in Besredka medium and the cultures were 13 days old on the day of injection. The bacterial sediment was collected by a pipett dried on filter paper weighed, ground in mortar and suspended in physiological saline t an amount of 1 mg/0 ml S spensions containing 10⁻ 10 etc. down to 10 mg/0 2 ml were prepared and 0.1 ml of suitable dil tions inoculated on each of ten Lowenstein-Jensen t bes. Calculated on the basis of the colony counts, the dose per animal in the group injected w th *M bovis* was 14 iable units and in the group injected with *M tuber losis* 700,000 iable unit

Q ntitat cult e Culture w performed using the whole spleen left liver lobe left lung and pooled lumbar mesenteric nd portal glands

The organs were removed by sterile autopsy and comminuted by grinding in mortar. About 1 ml diluted Sauton (one part Sauton medium and three parts water) was added dropwise. The suspension was treated with 4 per cent sodium hydroxide for 10 minutes and centrifuged for 15 minutes after which the supernatant was poured off. The sediment was neutralized with hydrochloric acid and 3 ml diluted Sauton added. From this suspension, designated 10, suspensions of 10^{-} 10^{-2} etc. were prepared with diluted Sauton and each of fiv Löwenstein-Jensen tubes was inoculated with 0.1 ml of suitable dilutions. The inoculated tubes were allowed to stand for 24 hours, the surface of the medium lying horizontally and then incubated at 37 C. The colonies of *M. bovis* and *M. tuberculosis* were counted after 8 and 6 weeks, respectively.

RESULTS

Autopsy Findings

Infection with M. tuberculosis. The portal, mesenteric and lumbar lymph glands were consistently enlarged during the interval from the 6th to the 63rd day. The glands, of a size up to that of hempseed, were first transparent, later they became of a whitish or slightly brownish colour and almost opaque. The content was soft and slimy but never caseous. Neither the liver nor the kidneys or spleen contained lesions, but after the 16th day the spleen was moderately enlarged (two to three times its normal volume). On the 19th and 63rd days a few non-caseous tubercles, were just visible in the lungs. In the last animal in th group (87th day) the only sign of the infection was the presence of a few small round, white nodules in the omentum. Two voles died spontaneously on the 63rd and 87th days, but the deaths were not caused by tuberculosis.

Infection with M. bovis. As from the 19th day the lymph glands in the abdomen were enlarged. Necrosis was visible from the 45th day when yellow foci appeared in the white content of the glands. As from the 60th day the size of the glands ranged from that of hempseed up to that of peas, the glands being completely caseous. Up to the end of the 2nd month, the liver spleen and lungs were normal. Only the last animal in the group which died on the 63rd day had developed generalized tuberculosis in the form described by *Wells* (1938) *viz.* caseous lymph glands everywhere in the abdomen and thorax and caseous tubercles in liver spleen and lungs.

A further three spontaneous deaths occurred during the experimental period, *viz.* on the 14th, 59th and 60th days, but it was only in the last two cases that death was caused by tuberculosis.

Results of Culture

The results are presented graphically in Figs. 1 and 2. The ordinate shows the logarithm of the number of viable units in the whole spleen, left liver lobe, left lung and lymph glands, calculated on the basis of the colony counts and the dilutions used for culture. Days after infection are plotted on the abscissa.

Infection with M. tuberculosis (Fig. 1). In all the organs, the number of viable units remained practically unchanged throughout the period. After the first month, there was a tendency towards a slight decrease in the number and the organs of the last animal in the group contained considerably fewer bacteria than those of the first animals.

Infection with M. bovis (Fig. 2). On the 12th day the lymph glands contained a few viable units, but otherwise culture from all organs was negative on the 6th 12th and 14th days. As from the 19th day tubercle bacilli were found systematically in the glands, liver and spleen and, from the 24th day also in the lungs. The number of viable units remained unchanged in the three animals killed on the 19th, 24th and 31st days. After that time, the number increased and towards the end of the period it was higher than in the corresponding animals infected with *M. tuberculosis*.

DISCUSSION

In his first experiments on field voles, *Griffith* (1937) found that a large dose of *M. bovis* injected subcutaneously provoked rapidly progressive tuberculosis. In animals injected with *M. tuberculosis* generally only few magnifi

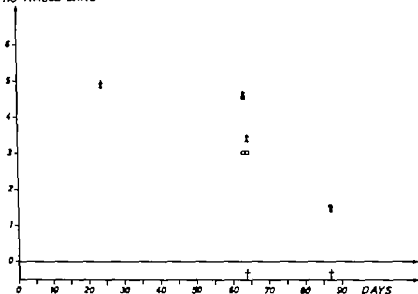
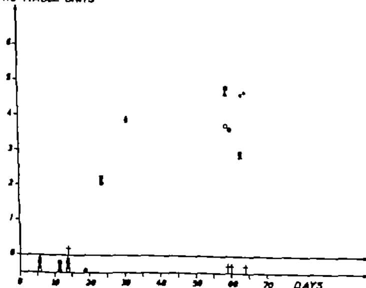


Fig. 1 Course of infection in *Microtus gr alis* (field vole) after intraperitoneal injection of 700 000 viable units of *M tuberculosis*.
Ordinate Logarithm of number of viable units in ● Portal, mesenteric and lumbar lymph glands. □ Left liver lobe. Δ Whole spleen. ○ Left lung *Abscissa* Days after injection



F g 2 Course of infection in *Microtus agrestis* (field ole) after intraperitoneal injection of 14 able units of *M bovis*. Symbols as in Fig 1

cant tuberculous lesions developed However one of the animals died late n the period from generalized tuberculosis

Using varying doses applied intraperitoneally *Wells* (1938) showed that a dose of 1 mg culture of *M tuberculosis* was required to induce a progressive infection, but only 10 mg of *M bovis* i.e the field vole is

at least 100,000 times more susceptible to *M bovis* than to *M tuberculosis*

Griffith (1939) resumed his experiments on subcutaneous infection with the two bacterial species, and arrived at a similar conclusion, and both *Wells* (1938) and *Griffith* (1939 1941) have recommended the use of field voles for differentiation.

The writer's results are in good accordance with those obtained by *Wells* In voles injected with 700,000 viable units *M tuberculosis* the bacteria did not multiply—at least not to any great extent—and the macroscopical processes which developed were insignificant. A considerably longer observation period would be required to ascertain whether the bacteria can provoke a fatal infection eventually or whether they gradually become destroyed and the lesion heals up Experiments hitherto performed (*Jespersen* 1974 1975) have shown that *M tuberculosis* persists for a long time in the organs of voles, and that the infection may flare up at a late stage and cause death.

In contrast, *M bovis* multiplied strongly in the field vole and doses as small as 14 viable units systematically provoked infection. With the exception of a transient inhibition in the 3rd to 5th week, due to immunity caused by the infection, multiplication of the bacteria continued throughout the course, resulting in the development of caseous processes which rapidly led to the death of the animals.

As regards its susceptibility to *M tuberculosis* and *M bovis* *Microtus agrestis* resembles the other members of the vole family so far examined, viz *Microtus arvalis* (common vole) (*Jespersen* 1975) *Clethrionomys g glareolus Schreb* (red mouse) (*Jespersen* 1954 *Hauduroy & Bize* 1957 *Bize* 1961) and *Arvicola terrestris* (vole rat) (*Jespersen* 1974)

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A COMPARISON OF ANTIGENIC STRUCTURE AND PHAGE PATTERN WITH BIOCHEMICAL PROPERTIES OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM SHEEP

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Oeding, P., Hájek, V. & Marsálek, E. A comparison of antigenic structure and phage pattern with biochemical properties of *Staphylococcus aureus* strains isolated from sheep. Acta path. microbiol. scand. Sect. B, 84 61–65, 1976.

Of 84 *Staphylococcus aureus* strains isolated from the anterior nares of healthy sheep and from the udders of ewes suffering from purulent mastitis the 89 per cent belonging to the C biotype contained agglutinogen A as well as polysaccharide Aβ. Ninety-five per cent of the C biotype strains were lysed by the bovine phage 78, the human phage set giving only weak reactions at RTD × 100. Two pigment-negative deficit variants as well as three unclassified strains gave similar results while three A biotype strains and one E biotype strain were definitely different. The close correlation between biochemistry, serology and phage typing substantiates the practical usefulness of the subdivision of *S. aureus* into biotypes.

Key words: *Staphylococcus aureus*; antigenic structure; phage pattern; biochemical properties.

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Staphylococci cause important infections in breeds of sheep. A primary epidemiological question is whether the infections have been evoked by strains specifically adapted to sheep or by strains transferred from man or other hosts. The answer depends on a thorough knowledge of the biological characteristics of ovine staphylococci.

In the present investigation the antigens and phage patterns of *Staphylococcus aureus* strains isolated from sheep have been compared with biochemical properties and the strains have been classified in biotypes (9, 15).

MATERIALS AND METHODS

In 15 farms of North Moravia and Central and Eastern Slovakia, 56 staphylococcal strains were obtained from the anterior nares of healthy sheep and 28 strains from the udders of ewes suffering from purulent mastitis. The 84 strains all produced heat stable nuclease and coagulated rabbit plasma, and were identified as *S. aureus*. The subdivision of the strains into biotypes (9, 15) was done according to coagulase activity in human and bovine plasma, the production of fibrinolysin, pigment, alpha and beta haemolysins, and the growth type on crystal violet agar. The detailed biochemical investigation was reported elsewhere (10).

For serological typing the live cell slide agglutination technique was employed according to *Oeding* (16) and *Haukenes* (11) using factor sera

TABLE 1 *Biochemical Properties of 84 S aureus Strains Isolated from Sheep*

Biotype		C	(C)	A	E	–
Number of strains		75	2	3	1	3
Deoxyribonuclease		75	2	3	1	3
Coagulation of plasma	rabbit	75	2	3	1	3
	human	75	2	3	0	3
	bovine	75*	2	0	1	3
Fibrinolysin		0	0	3	0	3
Pigment		75	0	3	0	3
Haemolysin	alpha	0	0	3	0	0
	beta	75	2	0	1	3
Crystal violet test	positive A	2	0	3	0	0
	negative C	73	2	0	0	3
	positive E	0	0	0	1	0

–: unclassified.
64 per cent positive after 24 to 72 h.
*: Pigment negative variants of biotype C.

μ μ b e h_μ h_p i s k k,k m 263-1 and *263-2* The antisera were obtained by immunization of rabbits with human *S aureus* strains and subsequent absorptions. Examination for polysaccharide Aα, Aβ Bα, Bβ and C, and for protein A, was performed using the technique described previously (17 18)

Phage typing was carried out with the basic sets of phages for typing human (25) and bovine (26) staphylococci. Strains resistant to RTD were retested at RTD × 100.

RESULTS

Of the total number of 84 strains 75 (89 per cent) belonged to the C biotype. They produced pigment and beta haemolysin but not fibrinolysin or alpha haemolysin, and grew with violet (negative) colonies on crystal violet agar (Table 1) All these strains coagulated both human and bovine plasma, the latter however by most strains (64 per cent) only after 24 to 72 h. Two strains, which formed no pigment, were classified as deficit variants of the C biotype.

Quite different properties were found in three strains which were classified as belonging to the A biotype and one strain which belonged to the E biotype. Finally three atypical strains were unclassified by the criteria used.

All the C biotype strains contained the *h_s* agglutinogen 67 strains giving very strong agglutination and 8 strains weaker agglutination One strain contained another agglutinogen in addition to *h* The pigment negative variants showed the same serological pattern as the typical C biotype strains (Table 2)

Of the three A biotype strains two contained the *h* agglutinogen, the reactions being weaker than in the C biotype strains. The third of these strains was non-typable. The one E biotype strain contained a strong *a_s* agglutinogen. The three strains which could not be subdivided biochemically contained the *h* agglutinogen

Polysaccharide Aβ was demonstrated in all but the one E biotype strain, the three A biotype strains also being found to contain poly

TABLE 2 *A t genic Patterns by Agglutination*

	Biotype C	(C)	A	E	–
h	74	2	2	0	3
h_s/b	1	0	0	0	0
	0	0	0	1	0
NT	0	0	1	0	0
Total number of strains	75	2	3	1	3

–: unclassified NT non-typable

TABLE 3 *Precipitinogens*

	Biotype				
	C	(C)	A	E	–
Poly Aβ	70	2	0	0	3
Poly Aβ, protein A	5	0	0	0	0
Poly A$_2\beta$, protein A	0	0	3	0	0
NT	0	0	0	1	0
Total number of strains	75	2	3	1	3

– unclassified. NT non-typable.

saccharide A_α (Table 3) Protein A was found in the three A biotype strains in addition to five strains (7 per cent) of the C biotype.

All the 75 C biotype strains were typable with phages, 55 (73 per cent) of them at RTD and 20 (27 per cent) at RTD × 100 (Table 4) Seventy-one strains (95 per cent) were sensitive to phage 78 of the bovine set, this phage being the only one active at RTD Using the basic set of human phages 44 strains (59 per cent) were typable at RTD × 100 These reactions often were weak and the patterns difficult to determine, the most usual being 3A/3C/42E/54/81 Four C biotype strains were lysed by bovine phages other than phage 78, three strains in addition being lysed by the same human phages as the bulk of the C strains.

The two pigment negative C biotype strains and one unclassified strain had the same phage pattern as the C biotype while two unclassified strains, in addition to the one E biotype strain, were non-typable. The three A biotype strains were lysed by human phages of group III at RTD and were clearly different from the other strains.

DISCUSSION

In the present study more than 90 per cent of ovine isolates biochemically belonging to the C biotype (10) were characterized by the presence of agglutinogen h_a polysaccharide A_β (β N-acetylglucosaminyl ribitol teichoic acid) and sensitivity to the bovine phage 78. These strains are substantially different both from human staphylococci, the great majority of which belong to the A biotype, and from animal staphylococci of other biotypes (9 15) The finding of the same biological properties in staphylococcal populations obtained from the mucous membranes of the upper respiratory tract, and from the udders or milk

TABLE 4 *Phage Patterns*

	Biotype				
	C	(C)	A	E	–
78	30	0	0	0	0
78(75 w)	1	0	0	0	0
78(42E/54w)	8	0	0	0	0
78(3A/3C/42E/54/81w)	17	1	0	0	1
78(3A/3C/42E/54/81/118/119w)	1	1	0	0	0
(78)(54w)	1	0	0	0	0
(78)(3A/3C/42E/54/81w)	9	0	0	0	0
(78)(3A/3C/42E/54/81/118/119w)	6	0	0	0	0
(118)	1	0	0	0	0
(118/119)(3A/3C/42E/54/81w)	3	0	0	0	0
42E/47/53/54/75/77(117)	0	0	3	0	0
NT	0	0	0	1	2
Total number of strains	75	2	3	1	3

In parentheses lysis at RTD × 100.
– unclassified. NT: non-typable.

specimens, leads to the conclusion that specifically *sheep-adapted staphylococci* most usually bring about mastitis in lactating animals.

In an earlier report (8) staphylococci from the anterior nares of healthy cattle were found to have biochemical properties similar to those of the ovine C biotype strains. These bovine strains also contained a strong agglutinogen *h* and polysaccharide A_β whereas the phage patterns of the two ecological groups of staphylococci were entirely different. Agglutinogen *h* has been reported to occur very frequently in animal staphylococci as a rule being the only agglutinogen of the human typing set demonstrable (13 19 21 22)

Attempts have been made to type ovine staphylococci by means of Pillet s sera. *Plommet & Wilson* (24) reported the agglutination of most strains in serum 16 and *Pillet et al.* (23) in serum 7 However the agglutinations observed were generally weak.

Polysaccharide A_β has been shown to be a wall component not only of C biotype sheep strains but also of human A biotype strains, swine and poultry B biotype strains, cattle C biotype strains and hare D biotype strains, whereas dog, pigeon and atypical swine and poultry strains contain other wall polysaccharides (5 6, 17) The presence of protein A in the human strains, but in only a few per cent of the ovine C biotype strains, is in accordance with earlier observations showing that this antigen is a characteristic component of human *S aureus* strains.

Sensitivity to phage 78 is undoubtedly a characteristic shared by the majority of ovine staphylococci. This was shown by *Davidson* (4) already in 1961 and confirmed in later studies (1 2, 14) In the present investigation an extraordinarily large number of phage type 78 strains were demonstrated. The present results also agree with earlier reports in the non-typability of ovine staphylococci with human phages at RTD and weak reactions with concentrated phages (1 5 7 12, 14 27) There seems to be a clear connection between the biotype determined on the basis of biochemical characteristics and its antigenic structure and phage sensitivity

The strains belonging to the A biotype were identical biochemically in their antibiograms, their content of wall polysaccharides and protein A, and in their phage type. They differed clearly from the C biotype strains. Their occurrence in the nasal mucosa of sheep kept on one farm strongly indicates transfer from a single human source.

The strain belonging to the E biotype corresponded in its properties to staphylococci obtained from dogs (20) Its isolation from sheep is an example of the possible transfer of staphylococci between various animal species.

In conclusion the ovine C biotype staphylococci are very homogeneous according to the characteristics tested for The excellent correlation between biotyping serological investigation and phage typing confirms the validity of the classification of *S aureus* into biotypes suggested by *Hájek & Maršálek* (9 15)

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FLAMING OF BIOPSY SPECIMENS FOR BACTERIOLOGICAL CULTURE SURFACE STERILIZATION AND EFFECT ON BACTERIA IN THE UNDERLYING TISSUE

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Lykkegaard Nielsen, M. Flaming of biopsy specimens for bacteriological culture surface sterilization and effect on bacteria in the underlying tissue. Acta path. microbiol. scand. Sect. B, 84 69–74 1976.

The effect of flaming (i.e. dipping biopsy specimens in alcohol and igniting them by drawing them rapidly through an open flame) on the reduction of bacterial surface contamination and on true bacterial concentrations in underlying tissue was investigated in different sizes of liver biopsy specimens in an experimental model suitable for quantitative and statistical calculations. Different degrees of surface contamination and of bacterial concentrations in underlying tissue were examined after a varying number of flaming procedures, the study comprising a total of 400 biopsy specimens. The flaming procedure repeated three times was able to eradicate a surface contamination of up to 10^4 *E. coli* per biopsy specimen, whereas a contamination of 10^6 *E. coli* per biopsy specimen was reduced to only 10^3 *E. coli*. Undesirable reduction in the true bacterial concentrations in tissue did not invalidate the method from a quantitative bacteriological point of view since the median reduction in bacteria per gram tissue in biopsy specimens of 1 cm³ size did not exceed a factor of 3 after three repeated flamings. It is concluded that in regard to surface sterilization the method is unreliable as a routine in postmortem bacteriological studies.

Key words bacterial contamination; surface sterilization postmortem bacteriology

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The possibility of bacterial contamination of biopsy specimens obtained during surgery (4 9) and of specimens obtained during autopsy (6, 14 17 19) or in animal experiments (2, 5 10, 18) has always been a problem in evaluating results of bacteriological investigations, and in the last decade the problem of bacterial contamination of biopsy specimens has been discussed especially in connection with sampling of postmortem tissues for bacteriological examination. Contamination may occur at any stage, i.e. during the obtaining of the specimen, during transportation to the microbiological laboratory during homogenization procedures and during dilution and culture of the homogenate With access to a well organized microbio-

logical laboratory the two first steps, i.e. obtaining the specimen and transportation would appear to represent the greatest and most uncontrolled danger of contamination. In postmortem examinations and in animal experiments, the hazards of contamination during biopsy have been dealt with in three different ways. *1* Biopsy has been performed under strictly aseptic conditions similar to those under which major surgery is carried out (10 14 17) *2* The surface of the organ in question has been seared in different ways, most often with a spatula heated to glowing and the specimen has been obtained with sterile instruments from the tissue beneath the seared surface (3 5 6, 7 15 19) *3* The specimen obtained under more or less *aseptic conditions has been dipped in alcohol* and ignited by drawing it rapidly through an open flame, this procedure being performed *once or repeatedly* (1 8, 11 16)

The method of flaming the biopsy specimen after dipping in alcohol is easily performed, and seems favorable from the point of view that the procedure can be performed in the microbiological laboratory just before *homogenization and is thereby* a precaution against contamination which has arisen during both biopsy and transportation procedures. The method raises three questions which *as far as* the present *author is aware* have never been studied on a quantitative scale 1) *To what extent does the method* involve an undesirable reduction in true bacterial concentrations in tissue? 2) Is it possible to eradicate any degree of surface contamination? 3) How does repetition of the flaming influence both bacterial concentration *in tissue and surface contamination?* The aim of the present investigation was to examine these questions on a quantitative scale in an experimental model suitable for statistical calculations.

MATERIAL AND METHODS

Experimental animals anaesthesia and surgical procedures. Rabbits of the New Zealand White strain, weight 3.2–3.5 kg, were used. Anaesthesia was accomplished with mebumal natrium (Nembutal®) approximately 40 mg intravenously per kg body weight. Hepatectomy was performed with all antiseptic precautions as previously described (10)

Bacterial test strain, homogenization and culture procedures. E. coli was used in all experiments. Homogenization of biopsy specimens in all experiments was always accomplished in two ml T 2 solution (13) in sterile glass grinders with sterile, electric-powered glass pestles, which matched the grinding tubes. It has previously been shown that this homogenization procedure does not reduce the number of viable bacteria (12) One half ml of the homogenate and one half ml of ten-fold dilutions were floated in duplicate onto blood agar plates. In experiments with low bacterial concentrations, the total homogenate was floated onto blood agar plates. Bacterial colony counts were made after 24 hours incubation at 35 C.

Flaming procedures: Biopsy specimens were *picked up with a needle-thin pincette heated to* glowing, dipped in absolute alcohol and ignited by drawing them rapidly through an open flame. In specimens flamed more than once the procedure was immediately repeated as soon as the flame on the biopsy specimen had gone out.

Reduction in bacterial concentrations in tissue. Different bacterial concentrations in the liver tissue were obtained by intravenous inoculation of different inocula of *E. coli*. The inocula used appear from Tables 1 and 2. Six sets of experiments *were performed, and in each set of experiments* hepatectomy was performed 30 minutes after bacterial inoculation, the liver being cut into 40 cubical biopsy specimens, which were randomly allocated into groups of ten. In three sets of experiments cubical specimens 0.125 cm³ in volume and in three sets cubical specimens 1 cm³ in volume were investigated. A total of 240 specimens were investigated, each set of experiments involving 40 specimens 10 unflamed biopsy specimens, 10 biopsy specimens flamed once, 10 biopsy specimens flamed twice and 10 biopsy specimens flamed three times. The weight of all biopsy specimens was recorded before homogenization, and in calculations of bacterial concentrations per gram liver tissue the dilution factors arising from both homogenization and serial ten-fold dilution were taken into consideration.

Contamination of biopsy specimens. For each set of experiments a rabbit liver was removed aseptically and cut into 0 125 cm³ cubes. Contamination *of specimens was accomplished by rolling the specimens* on a blood agar plate which had been floated 20 minutes previously with a suspension of *E. coli*. A total of 160 specimens were investigated in four sets of experiments with different degrees of contamination. Each set of experiments included 40 biopsy specimens: 10 unflamed specimens, 10 speci-

mens flamed once, 10 flamed twice and 10 flamed three times. The bacterial counts of the four different suspensions used for contamination and the degree of contamination obtained, appear from Table 3

Statistical calculations. In experiments on the effect of the flaming procedure on reduction in bacterial concentrations in the tissue, a small relative variation (range/mean) was observed, with a general tendency to increasing relative variance with increasing number of flamings. The numerical differences, however were so small that it was possible to perform two-way analysis of the variance according to inoculated dose of bacteria and number of flamings, using the logarithm of the mean of the bacterial concentrations within each experiment based on ten trials.

The effect of the flaming procedure on surface contaminated specimens was evaluated by nonparametrical tests: Mann-Whitney's rank sum test for unpaired differences was used in all cases except in comparisons between experiments, where more than one among ten biopsies in an experiment yielded zero bacteria. In those comparisons Fischer's exact test was used.

RESULTS

Reduction in bacterial concentrations in tissue following flaming of 0 125 cm³ and 1 cm biopsy specimens appears from Tables 1 and 2. From the bacterial concentrations in each experiment with unflamed specimens it is seen that regardless of the bacterial dose injected intravenously the hepatic uptake results in a quite even distribution throughout the liver tissue. Analysis of variance gave a constant reduction of 37 per cent (observed range 21–51 per cent) in bacterial concentrations in tissue following each flaming procedure in 0 125 cm biopsy specimens, regardless of the number of preceding flaming procedures. In 1 cm³ biopsy specimens, the corresponding reduction in bacterial concentrations in tissue after each flaming procedure was 22 per cent (observed range 6–40 per cent) In both sizes of specimens and in all sets of experiments (Table 1 and 2) the intergroup differences in bacterial concentrations between unflamed specimens, specimens flamed once, twice and three times were statistically significant at a common test level of 0.5 per cent. The difference in bacterial reduction between 0 125 cm³ specimens (37 per cent) and 1 cm³ specimens (22 per cent) was not statistically significant at the five per cent level $(0\,05 < p < 0\,10)$

The relative variance (range/mean) in unflamed specimens was <1 in flamed specimens also <1 in most cases, but generally increasing with increasing number of flamings.

Reduction in bacterial surface contamination following flaming of 0 125 cm³ cubical biopsy specimens appears from Table 3 The relative variance (range/mean) in unflamed specimens was 0 4–1 6 and in flamed between 2 and 8, generally increasing with increasing number of flamings. The reduction in number of viable bacteria between unflamed specimens and specimens flamed once, and

TABLE 1 *Reduction Bacterial Concentrations in Tissue Following Flaming of Cubical Liver Biopsy Specimens 0 125 cm³ in Size*

…cteria …oc …ated	Bacteria per ml blood at hepatectomy	Unflamed specimens	Bacteria per gram liver tissue — Specimens flamed: once	twice	3 times
10^4	0	124 (94 184)	78 (30–104)	54 (16–149)	34 (0–101)
10^6	0	2.2×10^4 $(1\,4 \times 10 \quad 3.3 \times 10^4)$	1.2×10 $(7\,1 \times 10^3–1.8 \times 10\ )$	$6\,0 \times 10^3$ $(79–1.2 \times 10^4)$	2.9×10^3 $(86–7.5 \times 10^3)$
$\times 10^8$	880	2.1×10^6 $(1.8 \times 10^6–2.8 \times 10^6)$	1.6×10^6 $(1\,3 \times 10^6–2\,6 \times 10^6)$	$1\,1 \times 10^6$ $(8\,0 \times 10^5–1\,4 \times 10^6)$	8.8×10^5 $(4.6 \times 10^5–1\,4 \times$

Each value tabulated is the mean for ten specimens with the range given in brackets

TABLE 2. *Reduction in Bacterial Concentrations in Tissue Following Flaming of Cubical Liver Biopsy Specimens 1 cm³ in Size*

Bacteria inoculated	Bacteria per ml blood at hepatectomy	Bacteria per gram liver tissue			
		Unflamed specimens	Specimens flamed		
			once	twice	3 times
4×10	0	240* (212–277)	205 (142–236)	166 (107–218)	155 (92–201)
1×10^7	0	5.4×10^4 $(4.7\times10^4–6.5\times10^4)$	4.0×10^4 $(3.1\times10^4–4.9\times10\ )$	3.0×10^4 $(2.0\times10^4–3.8\times10^4)$	2.6×10^4 $(1.8\times10^4–3.8\times10^4)$
3×10^8	1.1×10^3	3.4×10^6 $(2.6\times10^6–4.8\times10^6)$	2.5×10^6 $(1.5\times10^6–3.4\times10^6)$	2.0×10^6 $(1.3\times10^6–3.2\times10^6)$	1.2×10^6 $(7.0\times10^5–1.9\times10^6)$

* Each value tabulated is the mean for ten specimens with the range given in brackets.

between specimens flamed once and twice was always statistically significant, whereas this was not always the case between specimens flamed twice and three times (cf Table 3)

Calculated from the median (Table 3) one flaming of biopsy specimens caused the most constant and the most pronounced reduction, leaving about one per cent of the bacteria viable regardless of the degree of the surface contamination 0.8 per cent, 1.2 per cent, 0.3 per cent and 0.7 per cent, respectively in the four sets of experiments. Flaming twice caused a more variable reduction, leaving from 6 per cent to 63 per cent of the bacteria viable compared to the specimens flamed once, and leaving respectively 0.06 per cent, 0.07 per cent, 0.2 per cent and 0.08 per cent of the bacteria viable in the four sets of experiments compared to unflamed specimens. Flaming specimens three times reduced the median number of viable bac

TABLE 3 *Reduction in Surface Contamination Following Flaming of Cubical Liver Biopsy Specimens 0.125 cm³ in Size*

Number of bacteria on plate used for contamination	Number of bacteria per biopsy specimen			
	Unflamed specimens	Specimens flamed		
		once	twice	3 times
5.7×10^4	775 $(430–2.0\times10^3)$ §	7 (3–28) §	0.5 (0–6) †	0 (0–0)
1.1×10^6	1.0×10^4 $(6.4\times10^3–1.8\times10^4)$ §	116 (22–366) §	7 (0–115) ‡	0 (0–4)
7.7×10^6	7.8×10^4 $(6.4\times10^4–9.5\times10^4)$ §	276 (64–710) †	173 (0–255) †	8 (0–128)
5.1×10^9	3.3×10^6 $(2.1\times10^6–5.7\times10^6)$ §	2.2×10^4 $(6.4\times10^2–6.2\times10^5)$ †	2.6×10^3 $(21–2.4\times10^4)$ ‡	1.5×10^3 $(0–1.7\times10^4)$

Each value given is the median of ten specimens with the range given in brackets.

§ Difference between groups on each side of the sign significant ($p<0.01$)

† Difference between groups on each side of the sign significant ($p<0.05$)

‡ Difference between groups on each side of the sign not significant ($p>0.05$)

teria to zero in the two sets of experiments with the lowest degree of surface contamination, whereas respectively 0.01 per cent and 0.05 per cent of the bacteria in experiment no. 3 and no. 4 were still viable compared to the unflamed specimens.

DISCUSSION

The applicability of the present experimental model to quantitative and statistical evaluation of the effect of the flaming procedure depended on the ability to produce unflamed specimens within each set of experiments with only minor differences in bacterial content. The applicability was confirmed by the small range and the small relative variance in unflamed specimens in all types of experiments, the range of the relative variance being smallest in experiments on reduction in bacterial concentrations in tissue (Table 1 and 2). In these experiments, the bacteria in the liver tissue were virtually situated in the reticuloendothelial cells and was not merely a contamination from bacteria in the blood, since concentrations of bacteria in the blood were either zero or at least 10^3 fold less per ml blood than per gram liver tissue at the time of hepatectomy. This rapid bacterial takeup by the liver has been demonstrated by many authors, and will not be discussed further.

Although a strong effort was made to perform the flaming procedure uniformly, i.e. the dipping in alcohol and the rapid drawing through the flame, it was obvious that the flame of the specimen persisted for a varying number of seconds; this fact is probably reflected in the increasing relative variance (range/mean) in number of viable bacteria with increasing number of flamings, and many also account for the greater increase in relative variance in experiments on reduction of surface contamination (Table 3) compared to experiments on reduction in bacterial tissue concentrations (Table 1 and 2).

With regard to the undesirable *reduction in bacterial concentrations in tissue* the method, including flaming three times, seems acceptable in specimens of 1 cm³ size, since the median reduction of bacteria per gram tissue never exceeded a factor of 3 and since in any single specimens flamed three times the reduction never exceeded a factor of 5 compared to the median in unflamed specimens (Table 2). In small specimens (0.125 cm³, Table 1) flaming three times is not an acceptable method, since four out of ten specimens with a small median bacterial concentration *before flaming (122 bacteria per gram tissue)* were found sterile. Flaming twice, however, is acceptable, since the median reduction of bacteria never exceeded a factor of 4 and since the reduction in any single specimen (except one) never exceeded a factor of 9 compared to the median in unflamed specimens. The difference in bacterial reduction after each flaming between small specimens (37 per cent) and large specimens (22 per cent) is explainable if it is supposed that the flaming destroys bacteria to the same depth of the tissue independently of the size of the surface of the specimen.

Experiments on reduction of surface contamination were only carried out with one size of specimen, since no differences in principle were considered to exist between different sizes of specimens. Flaming performed three times could practically eradicate a surface contamination of 10^4 bacteria, whereas a median contamination of 10^6 bacteria was reduced to only 10^3 (Table 3). This must be considered a serious limitation of the method, since under most circumstances the possibility of contamination implies also that both the amount and the species of bacteria are unknown.

The method investigated in this report has not previously been studied on a quantitative scale. Discussion of previous qualitative studies will therefore be limited to the investigation of *Adamson* (1), to which reference in regard to reliability is made in recent major studies (16). In the thesis by *Adamson* the investigation on surface contamination included four lymph nodes dipped

three times in a '24-hour' bacterial culture and flamed two or three times the cut surface of the bi-sected glands (imprints on agar of the cut surface or fluid squeezed out) and pieces of the interior of the glands were found sterile, but the contaminated and flamed surfaces themselves were actually not cultured. In regard to undesirable reduction in bacterial concentrations in tissue, *Adamson* proved, that if 10^3 bacteria or more (the number estimated by me) were injected into lymph nodes, the bacteria could be qualitatively recovered from 15 of 16 glands investigated after two or three flaming procedures. There is no contradiction between the results of *Adamson* and those of the present investigation, but the two investigations are not comparable.

In conclusion the method is found acceptable for most purposes, including bacteriological autopsies, as far as involves the possibility of an undesirable reduction in the true bacterial concentrations in tissue. In regard to surface sterilization, the method is found unacceptable in general, also for bacteriological autopsies, but the method may prove useful in certain experimental situations, where the kind and degree of contamination are more under control.

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SOME FACTORS INFLUENCING THE HAEMOLYSIS OF *BORDETELLA BRONCHISEPTICA*

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Pedersen, K. B. Some factors influencing the haemolysis of *Bordetella bronchiseptica*. Acta path. microbiol. scand. Sect. B, 84 75–78, 1976

Strain-dependent variations in the ability of *Bordetella bronchiseptica* to produce haemolysis on solid media exist. Haemolysis is strongest at an acid reaction and will not take place if the reaction is too alkaline. Peptone and glutamine inhibit haemolysis and favour growth. The vigorous growth rapidly produces an alkaline reaction which inhibits the haemolysis.

Key words: *Bordetella bronchiseptica* haemolysis

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There is some confusion concerning the haemolysis of *Bordetella bronchiseptica* and it was therefore decided to study the factors which might influence the haemolysis of this organism.

MATERIALS AND METHODS

Strains. Eleven strains freshly isolated from different animal species were used. They had previously been used in serological studies (2).

Media. Meat infusion agar (MA) was prepared at the laboratory. Bordet Gengou medium (BG) was obtained from Difco. The peptone used was Bacto-Peptone (Difco). Defibrinated blood from calf, sheep and horse was used. The experiments using varying pH, peptone and amino acid were performed on media containing 10 per cent calf blood.

Measurement of pH on media with and without growth was performed as described by *Lautrop* (1). The media were flushed with 5 ml distilled water. After equilibration, the pH was measured in this water by a pH meter 28, Radiometer Copenhagen.

Changes in pH during growth were examined by seeding the half part on each of a series of plates. After incubation for 24 48 72, 96 and 120 hours at 37 C, a one cm broad zone closely adjacent to the seeded area was cut out and extracted with distilled water for subsequent measurement of pH.

Colony size and zones of haemolysis were measured by means of an AO Stereo Star Zoom Microscope.

RESULTS

Strains dependent variations in the haemolysis. On BG and MA at pH 7.0 and with 10 per cent calf blood it was possible to classify the strains into three groups according to the ability to produce haemolysis after incubation for 48 hours (Table 1). *Group I* was non-haemolytic on MA and weakly haemolytic on BG. Group III was strongly haemolytic on both media. The pig strains (75 2568, 3531 4389 4424) together with the rabbit strains (1024) and the mice strain (M 1) represented a group where haemolysis

TABLE 1 *The Haemolysis of* B. bronchiseptica *on Meat Infusion Agar and Bordet Gengou's Medium after 48 Hours Incubation (pH 7.0)*

Group	Strains	MA	BG
I	835 887	No haemolysis	Narrow appr. 0.1 mm β-haem.
II	75 2568 3531 4389 4424 1024 M 1	Very narrow (appr 0 1 mm) indistinct β-haem.	Narrow 0 1–0.2 mm β-haem.
III	361 R 1	Indistinct, appr 0.2 mm β-haem.	Clear 0.3–0.4 mm β-haem.

TABLE 2. *The Influence of pH on the Haemolysis of* B. bronchiseptica *on Meat Infusion Agar*

Group	pH interval with haemolysis	pH interval with max. haemolysis	pH interval without haemolysis
I	6.2–6.5	6.2–6.5	6.8–8.2
II	6.2–7.3	6.2–6 8	7 4–8.2
III	6.2–7.3	6.2–6.8	7.5–8.2

was weak to moderate on both media (group II) This grouping will be referred to in the following On BG with horse blood, the zones of haemolysis were broader than those with calf and sheep blood, this difference being less pronounced on MA. Generally the haemolytic zones were more distinct on BG than on MA.

The influence of pH on the haemolysis With a view to examining this influence, MA and BG were prepared with varying initial pH values. pH was adjusted by 1N NaOH and 1N HCl.

In one experiment with MA, the following pH values were obtained 6.2 6.5 6.8 7 1 7.3 7 4 7.5 7.8 and 8.2. Table 2 shows that the weakly haemolytic strains had the narrowest pH interval of haemolysis. It applies to all groups that maximal haemolysis was obtained at pH 6.2–6.8. All the strains were non-haemolytic at pH values exceeding 7.5

On BG (pH 6.3 6.9 7 1 7.3 7.6, 7.8 8.1 and 8.4) maximal haemolysis was observed at pH 6.9 At pH 6.3 an incomplete haemolysis around the colonies was observed. At this pH uninoculated areas of the plates were of a brownish colour Where confluent growth occurred the medium retained its red colour probably as the result of the rise in pH. The distinctness of the haemolytic zones was gradually reduced with increasing pH and, at pH 8 4 the strains were either non-haemolytic or incompletely haemolytic. On both media, the growth rate was not significantly influenced by the variation in pH.

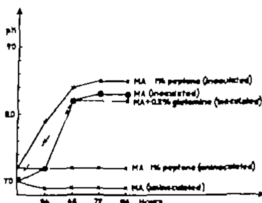


Fig 1 Changes of pH in *B. bronchiseptica* inoculated and uninoculated plates of meat infusion agar with and without peptone and glutamine.

The influence of peptone on the haemolysis. On BG and MA with one per cent peptone, all the strains were non-haemolytic. On these media (pH 7.0) using varying levels of *peptone* (0 0.2, 0.4 0.6, 0.8 and 1.0 per cent) the haemolysis of the weakly haemolytic group was inhibited by peptone concentrations exceeding 0.2 per cent. The remaining strains became non-haemolytic if concentrations exceeded 0.6 per cent. The diameter of colonies on peptone containing media were larger and they were less raised than those on the same media without peptone.

The changes in pH during surface growth of *B. bronchiseptica* on MA and BG with and without peptone are shown in Figs. 1 and 2. The same figures show the pH of uninoculated plates. It appears that the peptone containing media rapidly became alkaline. The difference between media with and without peptone was most pronounced after an incubation period of 24 hours. Moreover it appears that the pH of uninoculated BG is falling during incubation. Such a phenomenon was not observed in the case of MA.

In order to stabilize pH during growth, experiments were carried out with MA and BG containing 1 per cent peptone being poured on the surface of an agar (1 per cent) prepared of phosphate buffered saline (pH 7.0) During the initial 24 hours of incubation, pH did not rise significantly but after incubation for 48 hours, the reaction in buffered and non-buffered medium was of almost identical magnitude. On buffered plates, haemolysis with group II and III organisms was observed whereas there was no haemolysis on non-buffered media. The weakly haemolytic strains showed no haemolysis on buffered media.

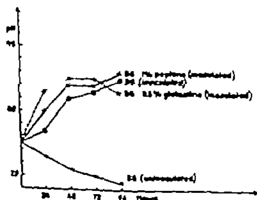


Fig. 2. Changes of pH in *B. bronchiseptica* inoculated and uninoculated plates of Bordet Gengou's medium with and without peptone and glutamine

Fractionation of peptone by filtration on Sephadex G-25 showed that components by which haemolysis was inhibited and growth favoured were eluted within the separation range of this gel, i.e. Mw 1,000–5 000

Dialysis of peptone containing media against media without peptone and *vice versa* showed that the growth-promoting principle inhibiting haemolysis was rapidly dialysable.

The influence of amino acids and amides on the haemolysis The influence on growth and haemolysis on MA of each of the following L amino acids and amides Alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, histidine, leucine, proline, tryptophan tyrosine and valine was studied by addition of 200 mg per 100 ml MA. Glutamine was the only compound which influenced growth and haemolysis. On MA, a concentration of 200 mg glutamine per 100 ml caused inhibition of haemolysis in all strains. On BG this concentration was only sufficient to inhibit haemolysis of the weakly haemolytic group, whereas higher concentrations (500 mg per 100 ml) were required to inhibit haemolysis of the group II and III organisms. The colonies on glutamine containing media were greater than those on media without glutamine.

Addition of glutamine to MA and BG caused the same rapid rise in pH during incubation as addition of peptone.

DISCUSSION

The present study revealed that strains of *Bordetella bronchiseptica* vary in their ability to produce haemolysis on solid media. From previous investigations it would appear that the heat labile antigenic structure of strains

with identical haemolytic features is identical (2)

The haemolysis was strongly dependent upon the pH of the medium. On BG maximal haemolysis was observed at pH of about 6.9. On MA, maximal haemolysis was seen in the range from pH 6.2–6.8. *Lautrop* (1) showed that the haemolysis of *B. pertussis* and *B. parapertussis* was strongest at an acid reaction (pH 6.0).

Addition of 1 per cent peptone to BG and MA completely inhibited the development of haemolysis of *B. bronchiseptica* and favoured growth. The vigorous growth rapidly produced an alkaline reaction and it is suggested that the rapid rise in pH, especially after 24 hours' incubation, may cause an inhibition of the haemolysis (Figs. 1, 2). This was further evidenced by the demonstration of haemolysis on peptone containing media layered upon a buffer agar with pH 7.0.

The gelfiltration and dialysis experiments suggested that the growth-promoting and haemolysis-inhibiting effect of peptone might be produced by low molecular organic compounds, probably peptides. Accordingly, the influence on haemolysis and growth of some amino acids and amides was examined.

Glutamine was the only one of the tested compounds which caused inhibition of haemolysis and increase of the colony size. It has previously been shown that glutamic acid is the main amino acid used as an energy source in the metabolism by *B. bronchiseptica* (3). Glutamic acid had not the same influence on growth and haemolysis as its amide. In other bacteria, the requirement of this amino acid for growth could not be satisfied by either glutamate or glutamine, but only by the two in combination (4).

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GROUPING OF BETA HAEMOLYTIC STREPTOCOCCI BY USING COAGGLUTINATION, PRECIPITATION OR BACITRACIN SENSITIVITY

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This study was made to compare coagglutination to precipitin test in grouping beta-haemolytic streptococci from clinical specimens and to investigate the accuracy of the bacitracin test in identification of group A streptococci. Results of grouping 126 strains with coagglutination and precipitation were identical in all except two cases. These two strains were nongroupable with precipitation but appeared as group B and C by coagglutination. When the distribution of group A, B, C and G streptococci in various clinical sources was investigated it was found that group B strains were the most frequently (41 per cent) isolated streptococci and even in isolates from pharyngeal swabs their proportion was 33 per cent. The accuracy of the bacitracin test in identification of group A streptococci was unsatisfactory as 26/62 (42 per cent) strains reported as group A by using this test were in fact group B, C or G streptococci. One of the reasons for this high number of false positives appeared to be the medium used for the preparation of the blood agar plates. In view of the frequent occurrence of non-A-streptococci in clinical specimens and high incidence of false positives in the bacitracin test it is suggested that this test should be replaced by a more efficient method of serological grouping.

Key words: Beta-haemolytic streptococci grouping coagglutination; precipitation bacitracin sensitivity

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Most laboratories engaged in diagnostic microbiological work use bacitracin test (*Maxted* 1953) for differentiating group A from other beta-haemolytic streptococci. This test is simple and well adapted to the routine work in a microbiological laboratory. However it differentiates only group A streptococci from the others and, moreover widely differing results have been reported about its accuracy even in this respect (*Maxted* 1953 *Chitwood et al* 1969 *Ederer et al.* 1972, *Moody* 1972 *Facklam et al.* 1974 *Pollock & Dahlgren* 1974) The recent emergence of serious infections, especially in neonates caused by group B streptococci, (Edit. 1974) has prompted our laboratory to search for a more suitable technique for streptococcal grouping This paper describes the study made to compare serological grouping by coagglutination, a method recently described by *Christensen et al.* (1973) to the classical

precipitin test. In addition, the accuracy of the bacitracin test in identification of suspected group A streptococci was investigated.

MATERIALS AND METHODS

Streptococcal strains were collected from the cultures of specimens sent to this laboratory. The specimens were from various clinical sources as shown in Table 1 mostly being throat swabs and samples from purulent infective sites. The samples were cultured on sheep blood agar plates (Blood Agar Base Oxoid Ltd, London, England) substituted with 7 per cent defibrinated sheep blood and incubated aerobically overnight. Colonies of beta-haemolytic streptococci were subcultured in 2 ml of Todd Hewitt broth (Difco Laboratories, Detroit, Michigan, USA) for serological grouping and tested for bacitracin sensitivity. Smears were prepared from the broth cultures for Gram staining. Known strains of group A, B C and G (kindly supplied by Prof *P Toivanen*, Dept. of Clinical Microbiology University of Turku, Finland) were included as positive controls. All the grouping methods were carried out blind relative to each other

Bacitracin Test

Colonies of beta-haemolytic streptococci were streaked with a loop on a sheep blood agar plate to obtain nearly confluent growth. After half an hour at room temperature a paper disc containing 0.2 IU bacitracin (AB Biodisk, Stockholm, Sweden) was placed on the agar plate and incubated over night after which the zones of inhibition were [illegible]red. According to the manufacturers instruc[illegible]ions inhibition zone diameters over 10 mm should [illegible]e obtained with group A streptococci.

In the experiment in which the effect of medium on the result of the bacitracin test was investigated, Blood Agar Base substituted with 7 per cent sheep blood was compared to Müller Hinton medium (Difco Laboratories) substituted with sheep or outdated human blood bank blood.

Grouping of Streptococci by Precipitation

To determine the Lancefield group, group specific substance from overnight cultures of streptococci in Todd Hewitt broth was extracted by autoclaving (*Rantz & Randall* 1955). Briefly overnight cultures of streptococci in Todd Hewitt broth were centrifuged at 1500 *g* for 10 min. The supernatants were discarded and 0.5 ml of saline was pipetted into the tubes. After autoclaving at 121 C for one hour the tubes were centrifuged again at 1500 *g* for 10 min. The clear supernatants were tested by double immunodiffusion in barbiturate buffered (0.15 M) 1 per cent agarose pH 8.1 on glass slides. The precipitates were clearly visible after incubation overnight at room temperature in humidified atmosphere. Commercial antisera were tested against control strains and A, C and G antisera from Difco and B antiserum from Behringwerke (Marburg, Germany) were found satisfactory and selected for use in this investigation.

Grouping of Streptococci by Coagglutination

The principle of this method lies in the use of a reagent consisting of staphylococci coated with group specific antibodies against streptococci. The binding of antibody to the surface of staphylococci occurs between protein A of the cocci and Fc part of the antibody leaving the antibody combining site free for immunological reaction. Commercial reagents, Phadebact Streptococcus Test (Pharmacia Diagnostics AB, Uppsala, Sweden) with anti-A, B, C or G group specific activity were used. One drop of each reagent was pipetted on a microscope slide. A drop of carefully mixed, overnight culture of streptococcus in Todd Hewitt broth was added to each drop of reagent and the slides were rocked gently for one minute. The slides were inspected against dark background and possible coagglutination recorded. In case all four reagents gave agglutination or the result was inconclusive for other reasons the cultures were trypsinized by adding 1 mg trypsin (Trypsin 1 250, Difco Laboratories) to 1 ml of broth and incubating at 37 C for 30–40 min. After that the coaggl tination test was repeated.

Fl orescent Antibody Te hnique

Smears of Todd Hewitt broth cultures were prepared on microscope slides and air dried. After fixing for 1 min in 95 per cent ethanol and drying, a drop of 1.5 dilution of fluorescein conjugated group A antiserum (Difco Laboratories) was added to each smear and incubated for 20 min. The conjugate was poured off and the slides rinsed three times for ten min in phosphate buffered saline, pH 7.2. After the last rinse in distilled water the slides were blotted gently and examined under UV microscope with oil immersion.

RESULTS

105 strains of unselected beta haemolytic streptococci were collected from successive clinical specimens. The clinical source of these strains and the distribution of the serological groups is shown in Table 1 Group B streptococci were isolated in as many as 41 per cent of all samples, even those isolated from the upper respiratory tract had a pro-

TABLE 1 *Clinical Sources of the Streptococcal Strains and Distribution of Serological Groups*

Source	A		B		C		G		Total
	n	%	n	%	n	%	n	%	n
Upper respiratory tract	25	43.1	19	32.8	9	15.5	5	8.6	58
Lower respiratory tract	2	14.3	9	64.3	0		3	21.4	14
Wounds and exudates	10	30.3	15	45.5	4	12.1	4	12.1	33
Total	37	35.2	43	41.0	13	12.4	12	11.4	105

TABLE 2. *Comparison of Coagglutination and Precipitin Test in Serological Grouping of Streptococci*

		Precipitation					
		A	B	C	G	Ng*	Total
Coagglutination	A	37					37
	B		53			1	54
	C			14		1	15
	G				18		18
	Ng					2	2
	Total	37	53	14	18	4	126

Ng = Non-groupable.

portion of B streptococci as high as 32.8 per cent.

To compare the technique of coagglutination with the precipitin test, altogether 126 strains were collected and the grouping performed independently from each other. The results of the series are shown in Table 2 which demonstrates good agreement between the two methods. In all cases the correct grouping of group A streptococci was verified by fluorescent antibody technique. Discrepancies existed in 2/126 cases. One strain was grouped as B by coagglutination but reacted in precipitation test with both A and B antisera repeatedly. In the fluorescent antibody test with group A antiserum this strain remained negative. The other divergent strain was group C, according to coagglutination method, but it appeared to be non-group assignable with precipitation.

The performance of the coagglutination method was assessed first by analysing the need for trypsinization of different streptococcal groups (Table 3). It appeared that about 95 per cent of B streptococci were groupable without prior trypsinization, whereas, in 60 per cent of other streptococci trypsinization was needed. This was mainly due to granular growth of the strains in Todd Hewitt broth. Another point of importance in assessing the performance of the coagglutination method, was the occurrence of non specific agglutination. This disadvantage was minimized or eliminated in a proportion of the cases by trypsinization but quite often group A streptococci still gave weak agglutination with C-reagent or group G strains reacted weakly with B-reagent. Again, most

TABLE 3. *The Need of Trypsin Treatment of Streptococcal Strains before Coagglutination*

Result obtained	A		B		C		G		Total	
	n	%	n	%	n	%	n	%	n	%
without trypsin treatment	15	40.5	50	94.7	7	50.0	5	27.8	77	63.1
after trypsin treatment	22	59.5	3	5.7	7	50.0	13	72.2	45	36.9
Total	37		53		14		18		122	

variant is provided with membrane invaginations that are not found in *cp* variants (15 16 17 18) Other differences are concerned with sensitivity to drugs. The cp^+ variant is more susceptible to the mutagens nitrosoguanidine ethidium bromide and acriflavin (12 13) as well as to some antibiotics (*Jyssum* unpublished results) Finally there are differences concerned with chromosome replication. It has been proposed that the change from *cp* to *cp* is followed by a change in the replication origin (10 12)

A transformable culture of *Bacillus subtilis* contains a small proportion of phenotypically competent cells. The fraction ranges from a few per cent to probably no more than 20 per cent (7 28) Several workers have isolated the competent fraction of the population by gradient centrifugation. In such technique, the population separates into two major components, the lighter of which contains the cells that are capable of incorporating transforming DNA (1 7 25 26 27) It has also been shown that, before acquiring competence, *B subtilis* cells pass through a precompetent phase starting 90–180 min before the appearance of competence (3) The precompetent state involves a changed pattern of cellular synthesis which is characteristic of a low growth rate a decreasing rate of DNA synthesis and decreasing ratio of RNA to protein synthesis (8)

In this paper sucrose gradient centrifugation has been used to examine the phenotypes of the *N meningitidis* competence variants. First the *cp* and *cp* variants have been analysed for any difference in sedimentation velocity The technique has later been extended to a further examination of the *cp* variant in order to find out whether a genotypically competent culture may be separated into components which differ in their capacity to be transformed otherwise observed in competent cultures of *B subtilis*.

MATERIALS AND METHODS

Bacterial strains. The following mutants from the *N meningitidis* Strain M1 of serogroup B was generally used: M1-6 *his pro* M1-8 *his arg* and M1 18 *his gly* (10) *Genetic competence in transformation* has been indicated by the symbol cp^+ and incompetence by *cp* (13, 22) Growth requirements and competence in transformation are controlled as described previously (13 22)

Media and growth. Blood agar plates and Heart Infusion Broth (HIB Difco) agar plates were used as solid complete media. Complete fluid medium was HIB. The basal media were those used before (9) Growth was performed and followed by measuring absorbancy or colony-forming units (CFU) as previously described (14)

Preparation of DNA Transforming DNA was prepared by a modification of the Marmur procedure (23) Streptomycin resistant DNA was isolated from a single-step, high-level resistant mutant (10)

Transformation system. Unless otherwise stated in the individual experiments the following transformation system was used (19) 0 7 ml HIB with 0 005 M $CaCl_2$ 0 2 ml receptor cell suspension containing approximately 10^8 CFU per ml 0.1 ml transforming DNA in NaCl-citrate buffer (0.15 M NaCl + 0.015 M Na_3 citrate, pH 7 4) Transformation took place at 37 C, and was terminated after the desired time, usually 45 min by the addition of 0 1 ml deoxyribonuclease (DNase) giving a final concentration of 50 µg/ml Transformants were enumerated according t the technique previously used (10)

Exposure f recipient cells to different concentrations of sucrose The effect of tonicity variation by means of sucrose on the transformation efficiency was tested as described before (19)

Isotopic labelling The genetically competent (*p*) variants were usually labelled with glucose-3-T(n) A stock solution of glucose was prepared which contained 400 µg glucose and 1 2 × 10^7 cpm per 200 µl The cells were first grown overnight on blood agar plates supplied with 200 µl of the stock solution. They were then harvested from the surface and re-inoculated in 10 ml batches of HIB supplemented with 200 µl of the stock solution of H glucose, After growth from A approximately 0.200 to A around 0.300 the cells were collected in the centrifuge (4000 × *g* for 15 min) and subjected to gradient centrifugation. The incompetent variants were as a rule labelled with glucose-U-^{14}C by similar technique. A stock solution was prepared so that 200 µl contained 1.64 × 10 cpm and 360 µg glucose.

C ll fractionation by density gr dient centrifugation. The *N men ngitidis* competence variants were fractionated on sucrose gradients by a technique modified from that described by *Richardson & Leach* (25) Unless otherwise stated, the cells were grown in 10 or 12 ml batches of HIB t the exponential phase. The cells were concentrated 5-fold in 5 per cent w/v sucrose containing the salts of

the *N meningitidis* basal medium (per liter: K_2HPO_4 – 7 g KH_2PO_4 – 3 g NH_4Cl – 1.0 g; glutamic acid – 1.0 g Na_2SO_4 – 50 mg $Na_2S_2O_3$ – 50 mg $MgCl_2$ – 0.1 mg $CaCl_2$ – 0.05 mg $MnCl_2$ – 0.01 mg) (9) and 0.1 or 0.2 ml of the cell suspension was layered on 10 ml sucrose gradient (5–40 per cent w/v) which as a rule was prepared in the salts of the basal medium. The cells were centrifuged in a Sorvall centrifuge for 10 min at 4000 rev/min and 4 C (25) A hole was pierced in the bottom of the tube and three drop fractions were usually collected. The drops were collected in tubes containing 0.4 ml HIB with 0.005 M $CaCl_2$. In some experiments, refractive index was measured on odd-numbered fractions. These fractions were collected in distilled water. To determine the presence of bacteria labelled by ^3H or ^{14}C, aliquots of the fractions were measured by scintillation counting in a Packard Tri-Carb spectrometer Instagel™ for aqueous solutions (Packard) was used as scintillation fluid. The competent cells in the fractions were detected by exposure to DNA and enumeration of the transformants. In order to maintain an optimal number of cells for transformation (around 10^8 CFU/ml) the fractions collected where the bulk of the cells banded were diluted (in the transformation medium) before exposure to DNA. Two or three transformations were usually performed using different dilutions of each fraction throughout this region. Transformants were counted after incubation with 5 μg transforming DNA per ml. The number of viable cells (CFU) in the fractions was enumerated by plating on blood agar plates after serial dilution through HIB (15)

Chemicals. Glucose 3-T(n) as well as glucose

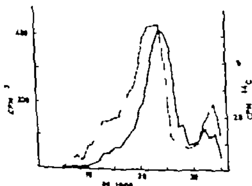


Fig 1 Sucrose gradient centrifugation of competence variants of the *N meningitidis* auxotroph M1-8 *his arg*. Solid lines genetically competent cells (cp^+) labelled with ^3H. Dotted lines genetically incompetent cells (cp^-) labelled with ^{14}C. Procedures as described under Materials and Methods.

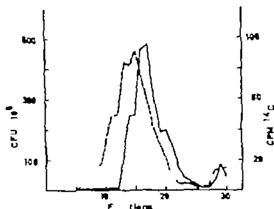


Fig 2 Sucrose gradient centrifugation of competence variants of the *N meningitidis* auxotroph M1-8 *his arg*. One part of ^{14}C labelled cp^- cells was mixed with 20 parts of unlabelled cp^+ cells. Solid lines colony-forming units (CFU). Dotted lines cp^- cells labelled with ^{14}C. Procedures as described under Materials and Methods.

U-^{14}C were purchased from The Radiochemical Centre, Amersham, Bucks, England. Other chemicals were those used previously (18, 19)

RESULTS

Separation of genetically competent (cp^+) *and genetically incompetent* (cp^-) *cells in sucrose gradients* Genetically competent variants (cp^+) were first labelled with ^3H, incompetent ones (cp^-) with ^{14}C, and mixed in very nearly equal numbers. The cell mixtures were run in sucrose gradients. Fig 1 is a drawing of the radioactivity distribution showing two bands for each of the competence variants. One dense band, and a far less dense band which concentrated just below the surface of the gradient. But the denser band containing the majority of the cp^+ cells sediments more slowly than the corresponding band from the cp^- cells.

Fig 2 records another type of experiment showing that a minority of cp^- cells labelled with ^{14}C was indeed separated from the bulk of the cell suspension which contained cp^+ cells.

In subsequent experiments, the labelling of the competence variants was changed, and the experiment illustrated in Fig 2 was re

peated using a majority of *cp* cells and a minority of labelled *cp* cells. The results were the same as those reported above. Competence variants from the auxotrophs M1-6 *his pro* and M1 18 *his gly* could also be separated by the same technique.

Effects of the sucrose and the cell density of the gradient on the transformability Several experiments were designed to test the effects of the passage through a sucrose gradient on the viability and the transformability of *N meningitidis*

The exposure to increased tonicity has been found to stimulate transformation in meningococci at osmolalities up to around 40 atmospheres (19) Fig 3 shows the transformability at various concentrations of sucrose such as those established in the sucrose gradients.

In *N meningitidis* concentrations of recipient cells well above 10^9 CFU/ml inhibit transformation. Fig 4 demonstrates that this reduction in transformation efficiency is virtually independent of the DNA concentra-

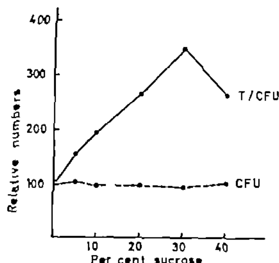


Fig 3 Effect of sucrose on the transformation efficiency in *N meningitidis* Suspensions of the auxotroph M1-8 *his arg* were exposed to various concentrations of sucrose in the salts of the basal medium for 20 min. Samples (0.1 ml) from each suspension were pipetted into HIB with 0.005 M $CaCl_2$. Transformation to Str r and plate counts after 20 min.

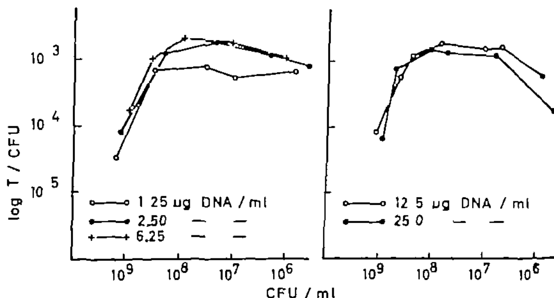


Fig 4 The effect of population density on *N meningitidis* transformation. The auxotroph M1-8 *his arg* was grown in HIB to the log phase. The cells were collected in the centrifuge and dense suspensions were prepared in the salts of the basal medium. These suspensions were diluted in the same medium, and aliquots were used as receptor cells in transformation to Str-r. The experiments were repeated with different concentrations of transforming DNA.

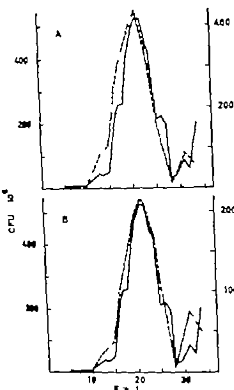


Fig. 5. Estimation of level of competence in sucrose gradient fractions. A genetically competent (*cp*) suspension from the auxotroph M1-8 *his arg* was fractionated on sucrose gradient. Solid lines cell counts (CFU) Dotted lines transformants (T) *Curve A* indicates the number of transformants actually measured, *Curve B* shows the number of transformants corrected for the effect of sucrose on transformability Transformation to Str-r

tion. When the cell concentration decreases below around 10^6 CFU/ml, the transformation efficiency also falls off Cell concentrations between 10^6 and 10 CFU/ml give practically the same efficiency With these densities of recipient cells, the DNA concentration curves for the *N. meningitidis* transformation system have been shown to be similar to those obtained from other transformable species, with a saturation level of around 2 µg DNA/ml (23, 24)

Transformability in sucrose gradients from genetically competent (cp) cells. The distribution of transformable cells in sucrose gradients from *cp* variants was next examined. Fig. 5 shows that the phenotypically competent cells form two peaks which seem to correspond to the bands of cells. The same result was obtained with cp^{+} cells labelled with ^{3}H (Fig 6) In Curve B of Fig 5 and Fig. 6, the numbers of transformants have been corrected to the values expected if sucrose had no effect on the transformation (19) The sucrose concentration through the gradient was established from readings of the refractive index (15 26)

Fig 7 shows that shortening of the time of exposure to DNA during transformation (by interruption with DNase) did not significantly shift the location of the peaks of transformants. The number of transformants is reduced to the extent previously found (11 24)

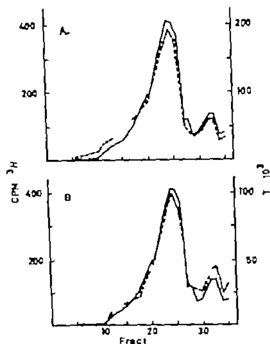


Fig 6 Estimation of level of competence in sucrose gradient fractions. A genetically competent (*cp*) suspension of the auxotroph M1-8 *his arg* was labelled with H and fractionated on a sucrose gradient. Solid lines density of cells indicated by ^{3}H activity (CPM) Dotted lines transformants (T) *Curve A* indicates the number of transformants actually measured, *Curve B* shows the number of transformants corrected for the effect of sucrose on transformability Transformation to Str-r

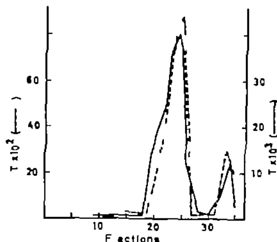


Fig 7 Transformation in fractions from a sucrose gradient. The genetically competent (cp^{+}) variant of the auxotroph M1-8 *his arg* was fractionated on a sucrose gradient. The cells in the fractions were used as recipient cells in transformation to Str r Solid lines interruption with DNase after 30 min. Dotted lines interruption with DNase after 15 min. (No correction for the effect of sucrose)

DISCUSSION

The *cp* variant sedimented a little more slowly than the cp^{-} variant in the sucrose gradients. This adds to the previously noted phenotypic differences between these genotypes. It may be suggested that the difference in sedimentation is connected with the observed differences in fimbriation or membrane structure (4 15 16, 17 18)

Comparison of transformability in fractions from sucrose gradients containing *cp* cells met with several problems, some of which have been dealt with in more detail in other publications (15 16 17 18, 19) The lethality caused by exposure to hypertonic concentrations of sucrose (15) was abrogated by addition of the salts of the basal medium and by collection of the fractions in complete medium with CaCl (15) Addition of minimal medium to the sucrose gradient also counteracted the loss of viability in *B subtilis* (25)

Renografin in concentrations up to 3.5 per cent slightly stimulated transformation of *B. subtilis* if present during exposure of the cells to DNA (7) In *N meningitidis* a stimulation by transitory exposure to sucrose in concentrations up to 30 per cent was found (19) It is conceivable that the difference in stimulation by sucrose in the concentration range covered by the peaks in the gradients (Fig. 5 and Fig 6) may be sufficient to move a corresponding peak of transformants to a heavier position. Therefore, the number of transformants in each fraction was tentatively corrected to the values expected if sucrose had no effect on transformability But the correction did not significantly change the location of the peaks of transformants.

Another factor which must be taken into consideration is the effect of the population density on transformation efficiency (25) The reason why transformation in *N meningitidis* is increasingly inhibited when the density of the recipient cell population is raised above approximately 10 CFU/ml is not altogether clear It does not seem to be caused by unsaturating concentrations of DNA. Other experiments indicate that the reduction in transformability is not caused by inhibitory substances released in dense suspensions, but is a direct consequence of the population density (*Jyssum* unpublished results) The exponential increase in cell mass of batch cultures of *N meningitidis* is also inhibited at approximately the same density and so is the abrupt enhancement in the rate of mass increase at regular intervals in synchronized cultures (14) Therefore, the reduction of the transformability may be a result of a reduction in the total metabolic activity of the recipient cells (24) By measurement of the transformability in the fractions, this practical problem was solved by dilution in order that the cell concentration in the fractions would be optimal across the gradient.

The transformable cells in the population of genetically competent (*cp*) variants of the *N meningitidis* Strain M1 did not form separate bands as found in *B. subtilis* transformation (1 7 25 26 27) *N meningitidis* transformation is characterized by an absorption of DNA much slower than that of other transformable species (11 24) and the usual transformation system allows exposure to DNA for 30 to 45 min (10 24) It is thus

conceivable that cells that were phenotypically incompetent might evolve competence during this period. But a shortening of the time of exposure (Fig 7) did not shift the location of the peaks of transformants. This substantiates the assumption that the phenotypically competent cells have indeed the same density as the rest of the population of *cp* cells. The experiments thus support the former notion that the phenotype actually capable of taking up extracellular DNA is expressed in the general population of *cp* cells during the exponential growth phase (19 24)

The author is greatly indebted to Miss *Lydia Oikle* for excellent technical assistance.

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Acta path. microbiol. scand. Sect. B, 84 93–100 1976

IN VITRO EFFECT OF METRONIDAZOLE ON THE ULTRASTRUCTURE OF *TRICHOMONAS VAGINALIS* DONNÉ

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Nielsen, M. H. *In vitro* effect of metronidazole on the fine structure of *Trichomonas vaginalis* Donné Acta path. microbiol. scand. Sect. B 84 93–100 1976.

The effect of metronidazole on the growth and fine structure of exponentially growing cells of *Trichomonas vaginalis* was examined. Cell division stopped about one hour after the addition of 1–4 μg/ml of metronidazole, and the cells were apparently arrested in the interphase. One to two hours after the addition of metronidazole, the movement of flagella and of the undulating membrane had decreased, but cell death had not occurred until 7–8 hours later The fine structure of the cytoplasm was changed 30 to 60 minutes after addition of metronidazole. Proportionally the number of polyribosomes had decreased whereas the number of single ribosomes in the cytoplasm had increased. The electron-density of the cytoplasmic matrix was higher in cells which were treated with metronidazole than in cells from control cultures. The findings of the present study indicate that the initial effect of metronidazol on *T vag* cells *in vitro* is an inhibition of cell multiplication as well as an impairment of protein synthesis.

Key words *Trichomonas vaginalis* metronidazole.

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The compound 1-β-hydroxyethyl-2 methyl-5-nitroimidazole (metronidazole) has proved to be an effective trichomonacidal agent (2, 4). It is effective on different protozoa and bacteria though its action is limited to the anaerobes or facultative anaerobes (14 16).

To a certain extent, the mode of action of the compound is recognized. *Ings et al* (8) found that the nucleic acid synthesis of *T vag* cells was strongly inhibited by metronidazole in low concentrations. *Fried & Fried* (6) and *Edwards & Mathison* (5) observed that metronidazole in high concentrations blocked the electron transfer mechanisms which normally lead to H evolution. *Ings et al* (8) concluded, however that both types of mechanisms probably are active at the same time.

Metronidazole-induced damage to *T vag* cells *in vitro* has been studied previously by electron microscopy (15). However the results obtained failed to provide information about the sites of the initial cell damage and to give additional morphological data on the previously mentioned biochemical effects. It is the aim of the present investigation to search for any early changes in the fine struc

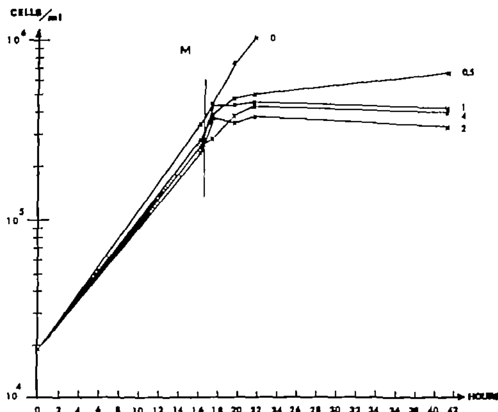


Fig. 1. Numbers of *T. vag.* cells plotted against time in a semilogarithmic scale. The growth curves demonstrate the effect of different concentrations of metronidazole on exponentially growing cultures of *T. vag.* Metronidazole in final concentrations of 0.5 µg/ml (0.5), 1 µg/ml (1), 2 µg/ml (2) and 4 µg/ml (4) was added at time M. Metronidazole in the concentration of 0.5 µg/ml alters the generation time of the *T. vag.* culture from approximately 4 hours to more than 24 hours, while higher concentrations of metronidazole completely stop cell division. The growth curve for a culture without metronidazole is marked 0.

ture of the *T. vag.* cell after *in vitro* treatment of exponentially growing cultures with metronidazole.

MATERIAL AND METHODS

T. vag., strain no. 1711, Neisseria Department, Statens Serum Institut, was maintained for more than one year in axenic culture by subcultivation in Diamonds medium (3). For this particular strain, the minimum inhibitory concentration of metronidazole *in vitro* was determined to be 1–2 µg/ml (1), i.e. no viable *T. vag.* cells were found in cultures which were incubated for 48 hours with metronidazole in this concentration (13).

Exponential Growth

The cells were grown in Diamonds medium without agar in an atmosphere of 95 per cent N and 5 per cent CO_2. This atmosphere was obtained by connecting each culture vessel to a rubber sack filled with the N_2/CO_2 gas mixture. The cultures were continuously agitated by means of a magnetic stirrer, and the culture vessels were provided with a separate glass pipe for repeated sterile sampling of *T. vag.* cells.

Cell counts were made in duplicate on 1 ml culture samples using a Coulter Counter model F with a manometric volume of 0.5 ml and with a 100 µm aperture. Before counting, the samples were diluted 1:20 with a 1 per cent formaldehyde solution in normal saline.

Metronidazole was added at a time during exponential growth when the cultures contained approximately 250,000 cells/ml. The growth rate and cell motility was followed for 7 hours and, in some experiments, for 22 hours after addition of metronidazole to final concentrations of 0.5 µg/ml, 1 µg/ml, 2 µg/ml and 3 µg/ml or 4 µg/ml. Cell

Figs. 2–7 The electron micrographs show sections of *T. ...ag* cells embedded in Vestopal-W. Sections were poststained with uranyl acetate and lead salts. For details of preparation see reference (11). The bar on each micrograph represents 0.25 μm.

Figs. 2 and 3 Micrographs of areas of *T. ...ag* cells from untreated exponentially growing cultures. The free ribosomes in the cytoplasm are mostly present as polyribosomes (RI). The cytoplasmic matrix (M) consists of flocculent material of varying electron-density. A nucleus (*N*), some of the perinuclear endoplasmic reticulum (*ER*), cross-cut costa (C) and some pericostal chromatic granules (CG) are also visible. The electron-translucent zones (GL) are possibly extracted glycogen granules. Magn. 60,000 ×

death was evaluated by examining unfixed, wet preparations stained with toluidine blue

Cells were collected for electron microscopy immediately before, and at intervals from 30 to 100 minutes after the addition of metronidazole to a final concentration of 4 µg/ml.

Preparation for Electron Microscopy

The cells were centrifuged (900 *G*) for 5 minutes and the pellet was fixed for 30–60 minutes in a 1 1 dilution with distilled water of the Karnowsky fixative (10) After a brief wash in 0._ M sucrose in 0.1 M cacodylate buffer pH 7._, the cells were embedded in melted agar at 45 C. Agarblocks containing cells were fixed in 1 per cent osmium tetroxide, dehydrated through ethanol and 100 per cent epoxypropane and then embedded in Vestopal W They were thin sectioned and stained for examination in the electron microscope as previously described (11)

Electron microscopy was performed with a Sie mens Elmiskop I A electron microscope.

RESULTS

Growth Motility and General Appearance of Cells

Within two hours the addition of metroni dazole to a final concentration of 0.5 µg/ml brought about a change in the generation time of the *T vag* cell cultures from approx imately 4 hours to more than 24 hours (Fig. 1) After 22 hours of incubation with ... ... zole, cell motility and viability re ained unchanged, but more than 50 per ..nt of the cells displayed evidence of in-..omplete cell division They had more than one nucleus and more than one mastigont.

Within one hour metronidazole in a con centration of 1 µg/ml had completely arrested cell division (Fig 1) and within 7 hours the movement of the flagella and the undulating membrane was markedly reduced. After 22 hours of incubation only a few per cent of the cells were still actively moving and about 70 per cent were dead. When examined by phase contrast microscopy the non-motile living cells were seen to be ovoid with a highly refractile periphery and with a cytoplasm of higher density than the cytoplasm of untreated cells. Incubation with metronidazole in concentrations of 2 µg ml and 4 µg/ml inhibited division of *T vag* cells within one hour (Fig 1) and 80 to 90 per cent of the cells had ceased to move after one and a half hours. Cell death did not occur during the first 7–8 hours but was *complete* after 22 hours.

The general appearance of these immobile, viable cells was the same as *that of cells treated with 1 µg/ml* of metronidazole de scribed previously

Fine Structure

Within 30 to 60 minutes after the addition of metronidazole to a concentration of 4 µg/ml, polyribosomes in the cells of the culture were sparse and mainly single ribosomes (R, Figs. 4 & 5) *generally uniformly distributed* in the cytoplasm, were seen. However the total number of ribosomes was increased by 10–20 per cent. The perinuclear granular endoplasmic reticulum was sparse compared with that of untreated cells. The fine struc ture of the cytoplasmic matrix was also changed. In the untreated cells, the matrix (M Figs. 2 & 3) was formed by flocculent material of varying electron-density In metronidazole treated cells the matrix appeared homogenous and of greater density to elec trons (M Figs. 4 5 6, 7)

The fine structure of chromatic granules, nucleus, Golgi region mastigont organelles, filamentous cell coat, and pinocytotic vesicles was unchanged in cells which were treated *for 60* minutes with 4 µg/ml of metronidazole. However after the cells had been incubated with this amount of metronidazole

Figs. 4 and 5 Micrographs illustrating areas from two *T vag* cells which were exposed to 4 µg/ml of metronidazole for 60 minutes. A high proportion of the ribosomes in the cytoplasm are present as single ribosomes (R) Compared with the cytoplasmic matrix of cells from control cultures, the matrix (M) of the metronidazole treated cells is more homogenous and more dense The limiting membrane of the nucleus (N) is indistinct and so are the membranes of the endoplasmic reticulum (ER) In Fig 5 a Aromatic granule is marked CG nd the axostyle AX. Magn. Fig. 4 62,000 Magn. Fig 5 35,000 ×

M
ER
4
CG
R
M
5

the DNA-dependent RNA polymerase (7) and metronidazole may correspondingly block RNA synthesis in *T vag* cells. The inhibition of messenger RNA synthesis could give rise to the dissociation of the polyribosomes, as has been observed in the present material. The period after initiation of treatment in which the morphological changes were first observed may be used for a rough estimation of the half life of the messenger RNA of the *T vag* cell. Thus determined, this half life seems to be at least 100 minutes.

The increased electron-density of the cytoplasmic matrix as well as the increased amount of free ribosomes in the cytoplasm may be due to a "dehydration of the trichomonad cell caused by the metronidazole treatment. These changes, that were observed by electron microscopy of the cells, may be the reason why such cells have an altered appearance when studied with phase contrast microscopy.

Whatever the mechanism may be the present investigation has shown that within few hours, metronidazole in therapeutic concentrations markedly reduced the protein synthesizing capacity of the trichomonad cell with concomitant deleterious effect on cellular function.

The author is indebted to Dr *Roderic Pontefract* Microbiology Research Division, Health and Welfare Ottawa, Canada, and to Mr *Aksel Birch Andersen* Biophysical Department, Statens Serum Institute, Copenhagen for valuable criticism during the preparation of the manuscript

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ULTRASTRUCTURE OF CELLS OF *TREPONEMA PERTENUE* OBTAINED FROM EXPERIMENTALLY INFECTED HAMSTERS

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Hovind-Hougen, K. Birch-Andersen, A. & Jensen, H. J. Skovgaard. Ultrastructure of cells of *Treponema pertenue* obtained from experimentally infected hamsters. Acta path. microbiol. scand. Sect. B, 84 101–108, 1976

Cells of *Treponema pertenue* Gauthier obtained by el tion from skin lesions and lymph nodes of experimentally infected hamsters were studied in the electron microscope by means of negative staining. The cells were also examined in thin sections of skin biopsies and lymph nodes. Fimbriae were observed on the negatively stained cells. Until now fimbriae have not been demonstrated on negatively stained cells of other species of *Treponema* but at present only one strain of *T. pertenue* has been studied in our laboratory. Otherwise, the ultrastructure of the *T. pertenue* cells was found to be very similar if not identical to, the substructural details observed in cells of *T. pallidum* and *T. cuniculi*. In thin sections of skin biopsies, treponemes were observed in the intercellular spaces between cells of the stratum basale and the stratum spinosum layers of the epidermis.

Key words: *Treponema pertenue* ultrastructure hamsters experimentally infected.

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Treponema pertenue is the etiological agent of the disease yaws. By darkfield and phase contrast microscopy cells of *T. pertenue* are impossible to distinguish from cells of *T. pallidum* the causal agent of syphilis. In addition presently available serological methods are unable to differentiate between the two treponematoses. By electron microscopy cells of *T. pallidum* and *T. cuniculi*—the causal agent of a venereal disease in rabbits—are found to be morphologically identical (5). Like *T. pertenue* these two treponemes are pathogenic and non-cultivable *in vitro*.

However electron microscopy carried out on cultivable, non pathogenic treponemal species has shown that it is possible to distinguish between cells of such species on morphological criteria (3, 4).

With these results in mind we felt that it would be worth-while to include *T. pertenue* in the series of treponemal species currently studied. In particular a comparison with the ultrastructure of the cells of *T. cuniculi* and *T. pallidum* was of interest for a possible distinction of cells of these two species from *T. pertenue* cells. The results of these studies are reported in the present paper.

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1
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2
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2
I
3
F



Fig 3 Fimbriae seem to protrude from the periphery of this cell (arrow) 90,000 ×

Fig. 4 Cytoplasmic tubules (CT) are seen in the middle region of a cell which was treated with *Myxobacter* AL-1 protease I for 10 minutes. Flagella (F) are also present. 160,000 ×

MATERIAL AND METHODS

T pertenue Gauthier was received in 1970 from Dr *A. Vaisman* Institut Alfred Fournier Paris, France. The strain was isolated in Nigeria in 1960 from a 10-year-old child with multiple papillomatae Hamsters were inoculated intradermally

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Figs. 1–4 show cells of *Treponema pertenue* negatively stained with 1 per cent ammonium molybdate pH 7 Figs. 5–9 all show sectioned material obtained from experimentally infected hamsters. The bar on each micrograph represents 100 nm unless otherwise stated.

Fig 1 These micrograph illustrate the regula waves presented by the cells after negath stal ing. Both cell has pointed end which learly show the three zones (1 3) (see text) Note the fine striation in zone 1 The three insertion points (I) of the flagell (F) are aligned Fimbriae are seen in Fig 2 (arrow) 90 000 ×

nd intrascrotally with exudate obtained from the edges of lesions (personal communication from Dr *Zah* WHO Geneva, Switzerland)

For this study the treponemes were propagated by intradermal inoculation in the groin of golden hamsters (*M socricet au atus*) as described by *Turner & Hollander* (9) The hamsters were 8 to 10 week old, random-bred, conventionally raised males from the animal farm of Statens Seruminstitut, Copenhagen.

Three to 5 weeks after inoculation, the skin lesions had reached diameter of 1 3 cm Skin with lesions and inguinal lymph nodules were then removed after the animals had been nesthetised with an intraperitoneal injection of 1 ml 0 6 per cent nembutal per 100 g body weight

P parations for V gative Staining

The lymph nodules or small pieces of skin with lesions were placed in al containing few drops of SMC (0 03 per cent sucrose in redistilled water with 0.01 M $MgCl$ nd 0 01 M $CaCl$) The tissue was minced with pai of scissors nd

flushed several times with the same few drops of SMC. The liquid with some tissue debris was transferred to a conical tube and left at room temperature for about 20 minutes to allow tissue debris to settle. The supernatant was carefully removed and examined by darkfield microscopy to see if the number of treponemes per ml was sufficient for further processing and examination in the electron microscope. A minimum number of about 3×10^7 treponemes per ml was necessary but, even if this concentration was attained, contamination with large numbers of blood cells and/or large amounts of tissue debris could make further processing useless.

Satisfactory preparations of treponemes were negatively stained as described previously (5)

Preparations for Sectioning

Skin lesions Small pieces of skin with lesions were cut out from the inguinal region of the hamsters and immersed directly in full strength *Karnovsky* fixative (7) The tissue was further processed as described previously (5)

Lymph nodes The inguinal lymph nodes were removed and immediately immersed in 3 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. Further processing of the tissue was performed as described for the skin biopsies (5)

All specimens were embedded in Vestopal-W

Ultrathin sections were obtained on an LKB III microtome and counter-stained with uranyl and lead salts according to the general routine of the laboratory Electron microscopy was carried out as described earlier (5)

For the present study approximately 350 recordings were studied.

RESULTS

Ultrastructure of Treponema pertenue

Negatively stained cells of *T pertenue* were regularly coiled with a mean wavelength of 1 1 μm and an amplitude of 0.2–0.3 μm. The cells were 8–15 μm long and about 0 15 μm wide. Sectioned cells also had a width of about 0 15 μm.

All negatively stained cells had pointed ends, and both ends of each cell were morphologically identical. At high magnification the terminal regions were found to consist of three zones, each with a characteristic structure and electron density (*Fig 1*) *Zone 1* consisted of the outermost tip of the cell which was rather electron dense. A substructure of *fine parallel* lines was frequently observed in this part (Figs. 1 and 2) Zone 2 consisted of the gradually widening part of the cell body which extended *from zone 1 to* the region where the flagella were inserted (Figs. 1 and 2) Zone 2 was about 0 18 μm long and was less electron dense than zone 1 Zone 3 was the region where the flagella were inserted (Figs. 1 and 2) This part had the same electron density as the rest of the cell.

The cells of *T pertenue* had three flagella inserted at each end (Figs. 1 and 2) with the flagellar insertion points aligned. The distance between the insertion points of the flagella was about 0 1 μm. The flagella from each end of the cell were wound around the cytoplasmic body and the flagella of each bundle interdigitated in the mid region.

Fimbriae were seen on many of the negatively *stained treponemes (Figs. 2 and 3) and* some of these fimbriae seemed to protrude from the cell periphery (Fig 3) The fimbriae were of varying length, generally from 1–5 μm. The width was 2–3 nm.

Cells treated with Teepol had lost only very few flagella, even if the treatment was prolonged to 10 minutes. The ultrastructure of the few flagella which were freed by Teepol treatment appeared to be very similar or identical to that of flagella isolated from cells of other *Treponema* species.

Treatment of *T pertenue* cells with *Myxobacter* AL-1 protease I for 10 minutes revealed bundles of cytoplasmic tubules in the organisms (Fig 4) Each tubule had a diameter of about 7 nm.

The ultrastructure of sectioned cells was studied in cells found in sections of the infected tissues. The treponemal cells were surrounded by a cytoplasmic membrane and an outer membrane. Both membranes were triple layered and each was 7–8 nm wide (Figs. 5 and 7) The flagella were situated in the interspace between the outer membrane and the cytoplasmic membrane (Figs. 5 and 7) *On most* cells the *outer leaflet of the* cytoplasmic membrane was obviously wider than the inner (Fig. 5) while on some cells the cytoplasmic membrane had a more symme-



Figs. 5–7 Sections of *T. pertenue* cells *in situ* in lymph nodes of infected hamsters. A transversely sectioned cell shows the flagella (F) in the interspace between the outer membrane (OM) and the peptidoglycan layer (P). On most cells the peptidoglycan layer is seen in close apposition to the outer leaflet of the cytoplasmic membrane (CM) so that this membrane generally has an asymmetrical appearance. Cytoplasmic tubules (CT) are situated in the cytoplasm adjacent to the cytoplasmic membrane and just underneath the flagella. Ribosomes (R) are present in the cell cytoplasm. V in Fig. 5 denotes a vacuole. Figs. 5–6 90,000 × Fig. 7 180,000 ×

trical appearance (Fig. 7). In the latter type of cells, the peptidoglycan layer (intermediate layer) was seen as a distinct electron dense line close to the outer leaflet of the cytoplasmic membrane and separated from this by a narrow electron lucent space (Fig. 7). In the cytoplasm of some of the sectioned cells small electron dense points were observed in apposition to the inner leaflet of the cytoplasmic membrane. These dense points were always positioned just underneath the flagella when these were seen cross-sectioned (Fig. 5). Most likely these points represent cross-sectioned cytoplasmic tubules. Ribosomes were evenly distributed in the cytoplasm (Figs. 5, 6 and 7) and in many of the cells membrane bound vacuoles were present (Fig. 5).

Observations on Infected Skin

The skin lesions were open ulcers of circular shape with centres at the inoculation sites and with diameters varying from 1 to 3 cm. The development of lesions was quite similar to the yaws-bejel type of reaction in hamsters (type Mh of *Turner & Hollander* (9)).

In the sections a moderate dilation of the intercellular space was observed between cells of the stratum spinosum and stratum basale layers of the epidermis. Otherwise the epithelial cells were reasonably well preserved and


M
D
D
8



Fig 9 High magnification of part of the field shown in Fig. 8. The treponeme is present in the inter space between two epithelial cells which are joined by the desmosome (D) The outer membrane of the treponeme is destroyed, but the cytoplasm of the organism and the asymmetrical cytoplasmic membrane is fairly well preserved. Ribosomes (R) a nuclear region (N) and flagella (F) are also seen. 90 000 ×

so were the desmosomes which joined them together A few leukocytes, in the main mononuclear cells and plasma cells, had invaded the epidermis and were seen between some epithelial cells (Fig. 8) The treponemes were observed only within intercellular spaces in the stratum spinosum and the stratum basale regions of the epidermis. They were often found apposed to the plasma membrane of epithelial cells (Fig 8) probably due to the narrow space between neighbouring cells of the epithelium. Treponemes were never seen intracellularly or between cells of the dermal layer nor were they found perivascularly or in the lumen of the small vessels examined. The outer membrane of the microorganisms was often partly or completely destroyed on cells found between epithelial cells in sections obtained from the biopsies of infected skin (Fig. 9)

Fig 8 Section of a skin biopsy from a region with lesions on an experimentally infected hamster. Treponemes (T) are present intercellularly in the stratum basale region of the epidermis. The tissue appears to be only slightly affected by the infection. Desmosomes (D) join cells which show well preserved cytoplasmic organelles, such as mitochondria (M) endoplasmic reticulum (ER) and tonofilaments (TF) The intercellular spaces are only moderately dilated, but occasionally leukocytes (L) are found here 16,000 ×

DISCUSSION

Cells of *T pertenue* presented ultrastructural details which were almost identical to those reported for cells of the species *T cuniculi* and *T pallidum* when these species were studied by comparable methods (1 2 5 6, 8) However negatively stained cells of *T pertenue* showed that the strain examined possessed fimbriae. The fimbriae were found consistently in our preparations of negatively stained cells, irrespective of whether the organisms were obtained from skin lesions or from lymph nodes of the infected hamsters. Recently we have re-examined all our micrographs of negatively stained cells of *T cuniculi* and *T pallidum* Nichols to ensure that fimbriae on cells of these species had not been overlooked. The examination of the *T cuniculi* material was carried out with particular care since the method of preparation used for these cells was identical to that used for the *T pertenue* material. Fimbriae were not found either on *T cuniculi* cells or on *T pallidum* cells. Thus, provided fimbriae can be demonstrated on cells of other strains of *T pertenue* than the one examined in this study the presence or absence of fimbriae may be of use for the differentiation between cells of *T pallidum* and *T cuniculi* on the one hand and those of *T pertenue* on the other However the fimbriae are difficult to

resolve in suboptimally stained preparations and at present we cannot conclude that it is possible to make a positive identification based on this particular morphological criterion.

In ultrathin sections, cells of the pathogenic treponemes *T cuniculi* *T pallidum* and *T pertenue* appear morphologically identical. The outer membrane of *T pertenue* was poorly preserved in biopsies from skin lesions, but was well preserved on cells in the lymph node material. The reason for this discrepancy is unknown to us. In this particular study we used formaldehyde-glutaraldehyde for the fixation of skin and glutaraldehyde for the lymph node material. Previously we have used formaldehyde-glutaraldehyde to fix skin of rabbits with *T cuniculi* infections and skin biopsies from patients with secondary syphilis. In these cases the treponemes observed all showed well preserved outer membranes. It seems unlikely therefore, that the different fixation methods used for the skin and lymph nodes of the infected hamsters can be responsible for the difference noted in the preservation of the outer membranes of the treponemes in the two tissues.

Cells of *T pertenue* were more resistant to treatment with Teepol and *Myxobacter* AL-1 protease I than the other pathogenic treponemal cells hitherto studied (2 5) Whether this can be regarded as characteristic for the species *T pertenue* or only for the particular strain used for this study is at present unknown.

Our studies on lymph nodes obtained from the experimentally infected hamsters will be reported separately

The authors wish to express their gratitude to Dr *H Aag Nielsen*, Department of Treponematoses, for his great interest in, and enthusiastic support of our work. We also thank Mrs. *J Berg*, Mrs. *H Rasn* and Mr *F Larsen* for their excellent assistance in electron microscopy and Miss *A. G Overgaard* and Mr *F Larsen* for expert photographic work.

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COMPARISON OF THE AGGLUTINATION AND THE CO-AGGLUTINATION TECHNIQUES IN T TYPING OF *STREPTOCOCCUS PYOGENES*

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Haug, R. H. Comparison of the agglutination and the co-agglutination techniques in T-typing of *Streptococcus pyogenes*. Acta path. microbiol. scand. Sect. B, 84: 109–111, 1976.

A comparison of two different methods in T-typing 200 Norwegian group A strains is reported. The traditional agglutination technique was compared with the co-agglutination method of Christensen and co-workers. Fifty-five strains could not be typed by either method. The typing results for 128 of the strains were in agreement. The most common T types were the following: 1, 4, 8, 12, 13, 28, and strains belonging to the T pattern 5/11/12/27/44.

Key words: Streptococcus group A; Norway; T-type classification; agglutination and co-agglutination methods.

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Group A streptococci contain two surface antigens, the M and T proteins. By means of these antigens, two systems of type classification have been established.

Identification of the M-antigen is based on Lancefield's precipitin reaction (3, 4, 6), while the T antigen is identified by agglutination (2).

The purpose of this paper is to compare the results of T type classification of 200 Norwegian group A strains by means of agglutination (2) and the co-agglutination method proposed by *Christensen et al.* in 1973 (1).

MATERIAL AND METHODS

200 Norwegian group A strains have been collected from different sources. Agar-plates containing 5 per cent horseblood were used for the primary isolation of the organism. Colonies exhibiting beta-haemolysis were tested for their sensitivity to 0.2 I.U. of bacitracin in paper disks (5) and classified by serological grouping according to Lancefield's precipitation method (3). The T typing was performed by means of agglutination (2) and co-agglutination (1).

The reagent for co-agglutination, consisting of a stabilized suspension of staphylococci coated with specific anti-T antibody, was prepared according to the procedure described by *Christensen et al.* (1).

The following T-typing antisera, used in our study, were purchased from Chemapol, Praha, according to the advice of Dr. *J. Rotta*.

Polyvalent antiserum pools. Constituting monovalent antisera.

T	1, 3, 13, B_{3264}
U	2, 4, 6, 28
W	5, 11, 12, 27, 44
X	8, 14, 25, Imp 19
Y	9, 2.., 23

Preparation of antigens for agglutination tests. The group A strains were inoculated in 10 ml

TABLE 1 *Distribution of Strains According to T-types*

T Types	*No. of* strains typed by agglutination technique	*No. of* strains typed by co-agglutination technique
1	11	11
3	6	5
4	24	25
2/4	1	
5		1
8	7	7
8/25	1	1
12	37	36
13	8	8
14	7	4
28	13	13
3/13/B_{3264}	7	1
13/B_{3264}	3	7
3/B_{3264}	3	
B_{3264}	6	8
5/11/12/27/44	11	11
non-typable	55	62
Total	200	200

Todd Hewitt broth and incubated at 30 C over night. The cultures were centrifuged and the sediment was suspended in 0.5 ml 0.2 M Tris HCL buffer pH 8.0 (1) Two drops of a 5 per cent trypsin solution in distilled water were added, and the suspensions were incubated at 37 C for one hour in order to make them smooth and stable for agglutination.

RESULTS

The results are shown in Table 1

The table shows the different T types and presents a comparison between the agglutination technique and the co-agglutination of *Christensen et al.* (1)

Out of 200 group A strains, 55 (27.5 per cent) were untypable using agglutination as compared to 62 (31 per cent) if co-agglutination were used. Strains registered as T types 1 8, 13 28 or belonging to the T-pattern 5/11/12/27/44 gave the same results by both methods. One strain could be typed only by co-agglutination. The latter technique indicated that it belonged to T type 5 The reaction was, however weak.

Out of 7 strains classified as T-type 14 by agglutination, only 4 strains were detected by co-agglutination.

The greatest difference, however appeared to be within the T-pattern 3/13/B_{3264}. One strain reacted positively in three of the monovalent sera, using co-agglutination, while 7 were found positive by agglutination. Three strains reacted with both the antisera 13 and B_{3264} and 3 strains with the antisera 3 and B_{3264} using agglutination. By co-agglutination, 7 strains gave positive results with the antisera 13 and B_{3264} none with 3 and B_{3264}.

Six strains reacted positively only with the serum B_{3264} using agglutination, 8 strains if co-agglutination were used.

One hundred and fortyfive strains could be typed by agglutination, 138 strains by co-agglutination. Applying the two methods, 128 strains reacted positively using both methods and gave results which were corresponding.

The most common T types among the strains were the following 1 4 8, 12, 13, 28, and strains belonging to the T pattern 5/11/12/27/44

DISCUSSION

In our study two different methods were used for T-agglutination. The technique used by *Christensen et al.* was compared with the traditional procedure. The typing results applying to 128 of our strains were in agreement. The two techniques did not result in different T type in any case.

To prepare the reagents for co-agglutination, very small amounts of specific antisera are needed. This is an important advantage as sera for the typing of streptococci are precious reagents.

T typing of group A strains is important, especially in epidemiological studies, as a method by which to classify strains which cannot be M typed The present study shows that the co-agglutination used by *Christensen et al.* may be an alternative to the traditional procedure.

My thanks are due to Dr *Kristian Odegaard* Bacteriology Department, National Institute of Public Health, Oslo, for streptococcus strains and to Dr. *J Rotta* Institute of Hygiene and Epidemiology Praha, for help and advice.

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5		1
6	7	7
8/25	1	1
12	37	36
13	8	8
14	7	4
28	13	13
3/13/B_{3264}	7	1
13/B_{326}	3	7
3/B_{3264}	3	
B_{3264}	6	8
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4	24	25
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5		1
8	7	7
8/25	1	1
12	37	36
13	8	8
14	7	4
28	13	13
3/13/B_{3264}	7	1
13/B_{3264}	3	7
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culture was dialysed against 1 per cent glycine and subjected to isoelectric focusing in a 110 ml column in density gradient of glycerol and an Ampholine pH gradient 3–10 (1 per cent w/v, LKB-produkter S-161 25 Bromma, Sweden) (16). The haemolytic activity was determined in serial twofold dilutions (17). Cytotoxic factor(s) was assayed on human diploid fibroblasts (15). Enterotoxin activity was assayed by three methods (i) rabbit intestinal loop test in two rabbits, (ii) rabbit skin test in two rabbits and (iii) adrenal cell test (7, 11, 14). Toxin samples were diluted with tris buffered saline. In the skin test, induration as well as blueing were necessary for a positive test. In the adrenal cell test, rounding of the cells was observed (7). A partially purified enterotoxin from *E. coli* strain 853/67 was used as positive control for every animal and tissue culture test. Protease, deoxyribonuclease (DNase), lecithinase and lipase activities were tested in sensitive plate assays (17).

An antiserum against *A. hydrophila* haemolysin (isoelectric point 5.5) with a neutralization titre of 64 U was used to inhibit the haemolytic activity tested (18). Rabbit antisera against choleragen were kindly supplied by Dr. *W. Unger*, Swiss Serum Institute, and an antiserum against *E. coli* strain 853/67 enterotoxin prepared by us (14) was used for inhibition experiments with Aeromonas enterotoxin.

Results and Discussion

All isolates of *A. hydrophila* except one produced extracellular haemolysin and showed cytotoxic activity on human embryonic diploid lung fibroblasts. The following extracellular enzymes were also detected: caseinolytic activity 9/11, DNase 11/11, lecithinase 10/11 and lipase 9/11. The results of the three different enterotoxin assays are summarized in Table 1. The higher sensitivity of the skin test and the adrenal cell test as compared with the classical ileal loop test for cholera and *E. coli* enterotoxin (11, 14) can probably explain why 6 out of the 11 strains were positive only in the first two tests.

TABLE 1. *Enterotoxin Activities in 11 Strains of* Aeromonas hydrophila *in Three Different Assay Systems*

Tests	
Loop, skin adrenal cell test positive	5/11
Skin and adrenal cell test positive	4/11
Skin test positive	1/11
Adrenal cell test positive	1/11

Strain 190a was selected for further studies. Table 2 shows the results of assays for enterotoxin and haemolysin on samples from different phases of growth. It can be noticed that the skin tests could not be readily interpreted for enterotoxic activity since dermonecrosis produced by haemolytic and cytotoxic factors interferes. However, after heating 1 ml test samples at 56 °C for 10 minutes the cytotoxic and haemolytic factors were inactivated and enterotoxic activity could be detected. Haemolysin also lyses the adrenal cells rapidly but enterotoxin can be detected also in this test system after heating at 56 °C for 10 minutes. Addition of specific antihaemolysin to test samples and incubation at 20° C for 30 minutes inhibited the haemolytic activity of test samples and permitted detection of enterotoxin by the skin test and the adrenal cell test. Simple fractionation of crude culture fluid on isoelectric focusing (16) gave the following isoelectric points (pI) for the different toxins: haemolysin pI 5.5 and 5.5, cytotoxic protein pI 5.5 and enterotoxin pI 5.2. Heating of crude enterotoxin samples and

TABLE 2. *Detection of Toxins in Culture Fluids of* A. hydrophila *Strain 190a after Different Cultivation Times*

Cultivation time (hours)	Loop test	Skin test: untreated	Skin test: 56 °C 10 min	Skin test: 80° C 10 min	Adrenal cell test: untreated	Adrenal cell test: 56 °C 10 min	Adrenal cell test: 80 °C 10 min	Haemolytic activity (HU/ml)
10	0	0	0	0	0	0	0	< 2
16	1.4	DN†	0	0	+	+	0	16
24	1.4	DN	0	0	+	+	0	64
32	1.6	DN	+‡	0	+	+	0	128
48	1.5 (0.8)§	DN	+	0	+	+	0	256

The numerical values indicate ml of fluid accumulated per centimeter of loop with a sample volume for each loop of 0.5 ml. (For details concerning the loop test see reference 14).
§ Sample volume for each loop 0.05 ml.
† DN: dermonecrosis with a zone ≥ 3 mm in diameter.
‡ indicates typical positive skin and adrenal cell test.

(PBS) TSRBC in 1 per cent rabbit serum, preabsorbed with NSRBC, in PBS. TSRBC were prepared as described by *Krupp* (5). Standard procedures were used for absorption of sera with NSRBC, TSRBC (3) guinea-pig kidney bovine erythrocytes (2) and treatment with mercaptoethanol (9).

The 31 sera giving anomalous Hepanosticon reactions were re-tested with this reagent after absorption by NSRBC, guinea-pig kidney and bovine erythrocyte suspensions. Sera still positive after absorption with NSRBC were absorbed with TSRBC, normal sheep serum and heat aggregated gamma-globulin. For the latter 16.5 per cent human gamma-globulin (Kabi) was diluted to 2.5 per cent with PBS and heated at 63 °C for 45 minutes. One volume serum was added to four volumes sheep serum or aggregated gamma-globulin, allowed to stand overnight at 4 °C and the supernatant used after centrifuging. To allow for dilution 50 µl absorbed serum was added to 0.5 ml Hepanosticon reagent. Absorbents were checked for lack of activity against Hepanosticon reagent. Twenty-five sera showing a rough edge to a negative Hepanosticon pattern were re-tested after absorption with NSRBC.

Sera were tested for anti-ruminant antibodies by double radial immunodiffusion against sheep serum using 0.7 per cent agarose in Tris-EDTA-NaCl buffer pH 7.6. A commercial latex slide method (RA Test, Hyland) was used for screening for RF.

Results

The 31 sera giving anomalous Hepanosticon reactions had anti-NSRBC agglutinin titres from 1/8 to 1/2048, geometric mean titre 91. The titres of the other 163 sera were up to 1/512, geometric mean 24. (Comparable Swedish sera had a geometric mean titre of 6). Anti-TSRBC titres were similar. Forty-nine of the sera with anomalous Hepanosticon reactions had anti-NSRBC titres of 1/16 or greater. Anti-NSRBC agglutinins in these sera were removed by NSRBC and guinea-pig kidney but not bovine erythrocyte absorption. Agglutinins against TSRBC, although in most cases diminished in titre, persisted in 28 sera. TSRBC absorption removed these: four sera needed up to three absorptions for complete removal of agglutinins. Absorption studies with the other 163 sera were similar. Of 124 sera with anti-NSRBC titres of 1/16 or higher there were 17 with agglutinins to TSRBC persisting after absorption with NSRBC. All were removed by TSRBC absorption. In all cases there was no agglutination of NSRBC or TSRBC after treatment of sera with mercaptoethanol.

Agglutination of Hepanosticon was absorbed from all but three sera by NSRBC or guinea-pig kidney whereas bovine erythrocytes had no effect. TSRBC absorption removed the three remaining reactions: treatment with sheep serum or aggregated gamma-globulin had no effect on agglutination. Sera showing an irregular edge to a negative Hepanosticon reaction gave normal negative patterns after NSRBC absorption.

Anti-ruminant antibodies, although found in nine sera, were not present in any giving anomalous Hepanosticon reactions. RF was present in 26 sera. Two of these gave anomalous Hepanosticon reactions. The anti-NSRBC titre of one was 1/512, the other 1/128. In each case agglutination was removed by NSRBC absorption. Treatment with sheep serum or aggregated gamma-globulin had no effect.

Discussion

The results show that although not in every instance associated with high titre, antibodies directed against NSRBC or TSRBC were probably responsible for the 31 false positive reactions with Hepanosticon. Agglutinins not removed by NSRBC absorption were absorbed with TSRBC whereas bovine erythrocyte absorption had no effect. Anti-sheep serum antibodies were not associated with anomalous reactions. In the two instances in which sera containing RF gave false positive reactions, the latter were absorbed with NSRBC but treatment with aggregated gamma-globulin had no effect.

The absorption studies, sensitivity to mercaptoethanol and reactions with TSRBC show the agglutinins in the Liberian sera to be identical to those described earlier from Nigeria (1). The reasons for the high levels of heterophile antibodies in many African sera are not clear. Some (1) have suggested a relationship with malaria, others (3, 4) do not agree. Whatever the cause, the practical implication is that Hepanosticon is not suitable for screening this population in view of the effort, time and expense necessary to eliminate anomalous reactions. These observations pertain also to other HA methods based on sheep erythrocytes.

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POSSIBLE FUNCTIONS OF PEPTIDE ANTIBIOTICS DURING GROWTH OF PRODUCER ORGANISMS BACITRACIN AND METAL(II) ION TRANSPORT

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The inhibitory effect of bacitracin upon growth of the producer strain *Bacillus licheniformis ATCC 10716* was dependent upon the presence of several different metal(II) ions, particularly Mn(II) Co(II) or Zn(II) ions. This supports our previous suggestion that the normal function of bacitracin during growth of the producer organism may be to promote the uptake of several divalent metal ions. Due to the striking similarity between the antimicrobial effect of bacitracin towards susceptible organisms and the effect of bacitracin towards the producer organisms *B licheniformis ATCC 10716* the possibility that the antimicrobial effect of bacitracin may be an induction of uptake of toxic amounts of metal ions is discussed. The possibility that peptide antibiotics may normally participate in ion transport during growth of producer organisms is also discussed.

Key words: Peptide antibiotics producer organisms bacitracin; metal(II) ion transport.

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Antibiotics are generally believed to be produced by cells that recently have stopped division (4 5 30, 31) These potent microbial metabolites are therefore not supposed to have any functions during active growth of producer organisms (4 5 30 31)

In contrast to this wiev we have recently reported that the peptide antibiotic bacitracin may be produced during active growth and may also have a function during active growth of the producer *Bacillus licheniformis* (15, 16, 17 18)

We have previously shown that bacitracin is inhibitory to growth of *B licheniformis ATCC 10716* only in the presence of excess Mn(II) ions (17) In low Mn(II) environments bacitracin even stimulated growth (17)

The normal function of bacitracin during growth of *B. licheniformis* may be to promote the uptake of several divalent metal ions (18) It is therefore possible that the inhibitory effect of bacitracin in the presence of excess Mn(II) ions may be due to an uptake of toxic amounts of this cation (17)

The aim of the present work was to investigate whether other divalent metal ions also

could influence the effect of bacitracin upon the producer strain *B. licheniformis ATCC 10716*

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. The bacitracin used has been described previously (17)

Organism

The bacitracin producing strain *Bacillus licheniformis ATCC 10716* was kept as a spore suspension at 4 C throughout the investigation.

Media and Growth Conditions

The complex RMO-medium has been described previously (18) Bacitracin was sterile filtered (Millipore) before added to the cultures. The following salts were added to the RMO-medium before autoclaving: $MgCl_2 \cdot 6H_2O$ $CaCl_2 \cdot 2H_2O$ $MnCl_2 \cdot 4H_2O$ $FeCl_3 \cdot 4H_2O$ $CoCl_2 \cdot 6H_2O$ $NiCl_2 \cdot 6H_2O$ $CuCl_2 \cdot 2H_2O$ $ZnCl_2$, $CdCl_2 \cdot 4H_2O$ The inoculum and growth conditions have been described previously (18)

Growth

Bacterial growth was measured as extinction at 650 nm (E_{650}) using a Spectronic 20 spectrophotometer

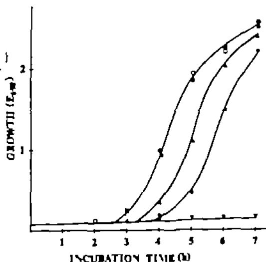


Fig 1 Effect of bacitracin upon growth of *B. licheniformis ATCC 10716* in the RMO-medium. Additions: nothing (○) 10 i. u. bacitracin/ml (■) 25 i. u. bacitracin/ml (▲) 50 i. u bacitracin/ml (●) 75 i.u. bacitracin/ml (▼)

Microbiological Assay and Detection of Bacitracin

Bacitracin was determined by an agar diffusion method described previously (14) Bacitracin was identified by thinlayer chromatography (t.l.c.) as described (14)

RESULTS

Bacillus licheniformis ATCC 10716 produced bacitracin during active growth in the complex RMO-medium. During early growth only small amounts of bacitracin were produced, whereas during later stages of growth a maximum titre of about 20 i.u. bacitracin was reached. The bacitracin produced was identified by t.l.c. as described elsewhere (14) We have previously shown that *B. licheniformis ATCC 10716* was able to produce bacitracin during active growth in the defined M2 medium (16)

B licheniformis ATCC 10716 was not particularly sensitive to its own antibiotic when grown in the RMO-medium. The addition of 10 i.u. bacitracin/ml had no effect upon growth (Fig 1) The addition of 25 i.u. bacitracin/ml produced only a slight inhibition of growth. As much as 75 i.u. bacitracin/ml had to be added in order to completely arrest growth during the first 6 hours of incubation (Fig 1) Neither was this strain particularly sensitive to bacitracin when grown in the M2 medium (17) This is in contrast to *Snoke & Cornell* (26) who reported that the early growth of *B licheniformis ATCC 10716* was markedly inhibited by 1 i.u. bacitracin/ml.

The addition of 100 mg/l of Mg(II) Ca(II) Mn(II) Fe(II) Co(II) Ni(II) or Zn(II) ions to the RMO-medium had no effect upon growth of *B licheniformis ATCC 10716* (Table 1) However if non-inhibitory amounts of bacitracin (10 i.u./ml) also were added growth would be seen to be completely inhibited in the presence of 100 mg/l of Mn(II) Co(II) or Zn(II) ions (Table 1) In the presence of 100 mg/l of Ni(II) ions a marked inhibition of growth was observed upon addition of 10 i.u. bacitracin/ml. The addition of 100 mg/l of

TABLE 1 *Effect of Divalent Metal Ions and Bacitracin upon growth of* B. licheniformis ATCC 10716 *in the RMO-medium*

Metal(II) ion added (100 mg/ml)	Amount of bacitracin added (i.u./ml)	Growth after 6 hours of incubation (E_{600})
non	0	2.15
non	10	2.10
Mg(II)	0	2.25
Mg(II)	10	2.10
Ca(II)	0	2.15
Ca(II)	10	2.15
Mn(II)	0	2.10
Mn(II)	10	0.15
Fe(II)	0	2.25
Fe(II)	10	2.05
Co(II)	0	2.00
Co(II)	10	0.15
Ni(II)	0	2.05
Ni(II)	10	1.05
Cu(II)	0	0.15
Cu(II)	10	0.15
Zn(II)	0	2.00
Zn(II)	10	0.15
Cd(II)	0	0.15
Cd(II)	10	0.15

Mg(II) Ca(II) or Fe(II) ions together with 10 i.u. bacitracin/ml had no effect upon growth of *B. licheniformis ATCC 10716* (Table 1).

The addition of 100 mg/l of Cu(II) or Cd(II) ions to the RMO-medium completely suppressed growth (Table 1). Table 2 shows that high concentrations of Mn(II) Co(II) Ni(II) or Zn(II) ions also could completely suppress growth of *B. licheniformis ATCC 10716*. Fe(II) ions were not particularly toxic to the cells. If more than 300 mg/l of Fe(II) ions were added to the RMO-medium, precipitation would occur. This resulted in an inhibition of growth.

Mg(II) and Ca(II) ions were essentially non-toxic to growth of *B. licheniformis ATCC 10716*. Very large amounts could be added without any inhibitory effect upon growth (Table 2). If more than 1000 mg/l of Mg(II) ions were added to the RMO-medium, precipitation would occur. This,

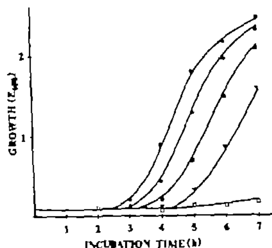


Fig. 2. Effect of bacitracin upon growth of *B. licheniformis ATCC 10716* in the RMO-medium supplemented with 200 mg Mn(II)/l. Additions: Nothing (○) 0.5 i.u. bacitracin/ml (●) 1.0 i.u. bacitracin/ml (■) 2.0 i. u. bacitracin/ml (▼) 5 i. u. bacitracin/ml (□)

however did not result in an inhibition of growth.

The addition of non-inhibitory amounts of bacitracin (10 i.u./ml) together with large amounts of Mg(II) or Ca(II) ions showed no inhibitory effect upon growth of *B. licheniformis ATCC 10716* (Table 3). In the presence of high amounts of Fe(II) ions

TABLE 2. *Effect of High Concentrations of Divalent Metal Ions upon Growth of* B. licheniformis ATCC 10716 *in the RMO-medium*

Metal(II) ion added	Amount of metal(II) ion (mg/l)	Growth after 6 hours of incubation (E_{600})
Mg(II)	300	2.25
Mg(II)	500	2.20
Mg(II)	1000	2.25
Ca(II)	300	2.25
Ca(II)	500	2.20
Ca(II)	1000	2.20
Mn(II)	300	0.15
Fe(II)	300	2.00
Co(II)	300	0.15
Ni(II)	300	0.15
Zn(II)	300	0.15

TABLE 3 *Effect of High Concentrations of Mg(II) C (II) or Fe(II) Ions and Bacitracin upon Growth of* B. licheniformis ATCC 10716 *in the RMO-medium*

Amount of Mg(II) ion added (mg/l)	Amount of Ca(II) ion added (mg/l)	Amount of Fe(II) ion added (mg/l)	Amount of bacitracin added (i.u./ml)	Growth after 6 hours of incubation (E_{650})
0	0	0	0	2.15
0	0	0	10	2.20
300	0	0	0	2.25
300	0	0	10	2.20
500	0	0	0	2.15
500	0	0	10	2.20
0	300	0	0	2.15
0	300	0	10	2.15
0	500	0	0	2.10
0	500	0	10	2.20
0	0	200	0	2.00
0	0	200	10	1.65
0	0	300	0	2.00
0	0	300	10	1.60

TABLE 4 *Effect of Low Concentrations of Cu(II) or Cd(II) Ions and Bacitracin upon Growth of* B. licheniformis ATCC 10716 *in the RMO-medium*

Amount of Cu(II) ion added (mg/l)	Amount of Cd(II) ion added (mg/l)	Amount of bacitracin added (i.u./ml)	Growth after 6 hours of incubation (E_{650})
0	0	0	2.20
0	0	10	2.25
10	0	0	2.20
10	0	10	2.15
20	0	0	2.05
20	0	10	1.80
30	0	0	0.65
30	0	10	0.15
0	5	0	2.20
0	5	10	2.10
0	7.5	0	1.40
0	7.5	10	0.55
0	10	0	0.35
0	10	10	0.15

only a slight inhibition of growth was observed when 10 i.u. bacitracin/ml was added (Table 3)

The addition of non-toxic amounts of Cu(II) or Cd(II) ions together with 10 i.u. bacitracin/ml showed no inhibition of growth (Table 4) However the inhibitory effect of these ions could be enhanced by the addition of bacitracin (Table 4)

In the presence of high but non-toxic amounts of Mn(II) ions, small amounts of bacitracin significantly inhibited growth of *B. licheniformis ATCC 10716* in the complex RMO-medium (Fig 2) We have previously shown that small amounts of bacitracin could inhibit growth of *B licheniformis ATCC 10716* in a defined medium supplemented with high amounts of Mn(II) ions (17)

DISCUSSION

Although antibiotics are not believed to have any function during growth of the producer organisms, it is frequently observed that antibiotics may be detrimental to the producer cells when added to the early growth phase (5 26, 32) It is suggested that the producer organisms are sensitive to their own antibiotics during growth because they normally do not produce antibiotics at this time of their life cycle (5 26)

However during the last years it has been increasingly apparent that many micro-organisms may normally produce antibiotics during active growth (2, 3 13 16, 17 18) Since many antibiotics may be produced during growth and since they also may have effects upon growth of the producer cells, we find it possible that these antibiotics may have a natural function during growth. Thus it is possible that many antibiotics may be naturally occurring primary metabolites and not secondary metabolites as suggested by others (4 30 31) In accordance with this view we have proposed that the peptide antibiotic bacitracin may be an external promotor in the transport of several metal(II) ions during growth of the producer strains of *B. licheniformis* by complexing both with the metal(II) ions and the cytoplasmic membrane (15 17 18)

The present work shows that bacitracin is strongly inhibitory to growth of the producer strain *B licheniformis ATCC 10716* in the presence of excess Mn(II) Co(II) Zn(II) or Ni(II) ions. This supports our previous suggestion that bacitracin may stimulate the uptake of several divalent metal ions during growth of *B. licheniformis*. The addition of bacitracin results in a rapid uptake of these ions and toxic concentrations are reached inside the cells. The four metal (II) ions mentioned are all toxic to growth of *B licheniformis* when added in great excess (Table 2)

Bacitracin was not inhibitory to growth of *B. licheniformis ATCC 10716* in the presence of large amounts of Mg(II) or Ca(II) ions. These two cations are not particularly toxic to micro-organisms. Mg(II) ions may be present in relatively high concentrations inside the cells, and may be an important cofactor for many enzymes. It is reported that the relative concentration of Mg(II) to Mn(II) ions in *B subtilis* during growth is 167 1 (7) Since bacitracin is able to complex with Mg(II) and Ca(II) ions (27) it is possible that bacitracin also promotes the uptake of these cations. However since Mg(II) and Ca(II) ions are essentially non-inhibitory to *B licheniformis ATCC 10716* (Table 2) an increased uptake due to added bacitracin will not be observed as an inhibition of growth.

Bacitracin could also influence the growth of *B. licheniformis ATCC 10716* in the presence of excess Fe(II) ions. However there was only a slight inhibition of growth in this case. Bacitracin is able to complex with Fe(II) ions (12) Thus the inhibitory effect upon growth may be due to an increased uptake of the ion. Since Fe(II) ions do not seem to be particularly toxic to cell growth, it is understandable why an increased uptake due to bacitracin only results in a slight inhibition of growth.

Cu(II) or Cd(II) ions were strongly toxic to growth of *B licheniformis ATCC 10716* Bacitracin seems to be unable to stimulate the uptake of these ions if they are present in low and non-toxic concentrations. However bacitracin could enhance the toxic effect of Cu(II) and Cd(II) ions (Table 4) It is reported that Cu(II) ions binds very strongly to bacitracin (12) It is possible that this may explain why bacitracin is ineffective in promoting the uptake of this ion. Cu(II) ions bind strongly to the bacitracin molecule to be effectively transferred to a transport mechanism in the cytoplasmic membrane. Similarly Cd(II) ions are reported to bind strongly to the surface of a bacterial cell (29) Thus bacitracin may be ineffective in carrying Cd(II) ions to the cytoplasmic membrane since this ion may be too strongly bound by the cell wall. However in the presence of excess Cd(II) ions (in relation

to the binding sites on the surface of the cells) bacitracin may increase the uptake of Cd(II) ions. This is supported by the observation that bacitracin markedly increases the toxic effect of Cd(II) ions. (Table 4)

We have previously suggested that the frequently observed inhibition of the early growth of producer strains by their own antibiotic may be due to the presence of excess metal ions during early growth (17). The present experimental work is in support of this suggestion since bacitracin markedly inhibited growth of the producer strain *B. licheniformis ATCC 10716* in the presence of excess of several divalent metal ions. *Snoke & Cornell* (26) reported that small amounts of bacitracin (1 I.u./ml) markedly inhibited early growth of *B. licheniformis* ATCC *10716*. Their medium contained large amount of Cd(II) ions (about 17 mg/l). This may be the reason why small amounts of bacitracin could inhibit growth of *B. licheniformis ATCC 10716* in their medium. Our results show that bacitracin markedly enhances the toxic effect of Cd(II) ions.

It is reported that the antimicrobial effect of bacitracin toward *Staphylococcus aureus* is dependent upon the presence of a divalent cation such as Mn(II), Fe(II), Co(II), Zn(II), Ni(II) or Cd(II) ions. The Ca(II), Mg(II) or Cu(II) ions could not influence bacitracin activity, whereas Fe(II) ions only slightly influenced (1). Thus there is a striking similarity between the antimicrobial effect of bacitracin and the effect of bacitracin upon the producer strain *B. licheniformis ATCC 10716*.

The antimicrobial effect of bacitracin towards susceptible organisms may be to disturb membrane structure by complexing specifically with the functional membrane lipid C_{55}-isoprenyl pyrophosphate (27, 28). The function of divalent cations may be to promote the formation of the complex between bacitracin and the functional lipid (27, 28). It is supposed that the binding of bacitracin to this membrane lipid may disrupt the membrane structure of susceptible organisms sufficiently to produce lethal permeability changes (27, 28).

Since the effect of bacitracin upon the producer strain *B. licheniformis ATCC 10716* may be to increase the uptake of several divalent metal ions by promoting the interaction between the metal(II) ions and the transport mechanisms in the cytoplasmic membrane, we find it possible that the antimicrobial effect of bacitracin towards susceptible organisms may be of a similar nature. By complexing both with metal(II) ions and membrane lipids bacitracin may bring the metal(II) ions in close contact with transport mechanisms in the cytoplasmic membrane. This may result in uptake of toxic concentrations of the metal(II) ions. This is supported by the observation that the antimicrobial effect is directly enhanced in the presence of metal(II) ions which usually are toxic in excess (Mn, Zn, Cd) whereas the non-toxic cations (Mg and Ca) have no influence upon the antimicrobial effect of bacitracin (1). Our suggestion is also supported by the observation that several different metal(II) ions (Mg, Co, Ni, Zn) may be translocated by the same transport mechanism (29). That the antimicrobial effect of bacitracin may be an induction of rapid uptake of toxic metal ions, is also supported by the observation that the metal chelator EDTA is able to completely suppress the antibacterial effect of bacitracin (1).

In contrast to the effect of antibiotics upon the producer organisms, very much knowledge has accumulated about the molecular basis of antibiotic action towards susceptible organisms (11, 21). The peptide antibiotic polymyxin is reported to interact specifically with the functional membrane lipid phosphatidyl ethanolamine leading to irreversible damage of membranes (13). Valinomycin and gramicidin may also attack membranes as indicated by their ability to induce ion transport across black lipid membranes (21, 23). Valinomycin by carrying monovalent cations through lipid barriers (23) and gramicidin by inducing the formation of waterfilled channels or pores in membranes

(21) These channels may be specific to certain monovalent cations (22) Many other antibiotics both of peptide and non-peptide type (alamethicin, enniatin, monensin, nigericin, monectin, nystatin) are supposed to exert their antibacterial effect by interaction with membranes (11 21 23, 24) These antibiotics have the interesting biological property to facilitate the movement of ions across lipid barriers and have been termed "ionophore antibiotics" (23) These "cation conductors" have been extensively used in model systems used to study biological transport through artificial and natural membranes (21 23 24)

Pressman (24) suggests that the ionophore antibiotics may be analogous to naturally occurring membrane components participating in ion transport in micro-organisms. He further supposes that natural membrane bound carriers would be expected to require additional properties beyond those shown by ionophores (24) The possibility that many antibiotics may be "anachronistic remnants of primitive transport systems" has also been mentioned (21)

The biosynthesis of bacitracin and gramicidin S have recently been described (8, 9 10, 19 20) These peptide antibiotics are synthesized by complex enzyme mechanisms involving unusually large numbers of catalytical functions. This interesting non-ribosomal biosynthesis of peptides has been termed the protein thiotemplate mechanism (20)

We find it unlikely that micro-organisms should construct complex enzyme molecules such as the bacitracin and the gramicidin S-synthetases which are able to produce peptides possessing remarkable biological properties, and that these peptides should only be "anachronistic relics" (21) or just serve a detoxification purpose after growth (6, 30 31) We have previously mentioned the possibility that many peptide antibiotics may participate in ion transport during growth of the producer organisms (18) This view is supported by the following observations

a) Peptide antibiotics have excellent metal binding properties (12)
b) they have the biological ability to interact with membranes and to alter membrane permeability
c) they may be produced during active growth,
d) all *Bacillus* species may produce small amounts of most peptide antibiotics (25)

Thus peptide antibiotics may be functional microbial metabolites which actually may constitute some of the molecular basis of cation transport in micro-organisms.

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GENETIC MAPPING OF THE K1 AND K4 ANTIGENS (L) OF *ESCHERICHIA COLI*

Non Allelism of K(L) Antigens with K Antigens of O8 K27(A) O8 K8(L) and O9 K57(B)

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Ørskov I., Sharma, V & Ørskov F Genetic mapping of the K1 and K4 antigens (L) of *Escherichia coli* Non-allelism of K(L) antigens with K antigens of O8.K27(A) O8.K8(L) and O9:K57(B) Acta path. microbiol. scand. Sect. B, 84 125–131 1976.

Escherichia donor strains having antigen K1(L) or K4(L) transfer these K antigens to recipient cells at a genetic locus (*kps A*) similar to that of K10(L) and K54(L) linked to *ser A* I crosses between the K10 donor strain and recipient strains O8 K8(L) O8.K27(A) and O9.K57(B) all recombinants which inherit donor K antigen also inherit K antigen of recipient. This result is interpreted as non-allelism between donor and recipient K antigens, and it is assumed that the structure of all polysaccharide K antigens of strains having O antigens O8 or O9 whether termed L, A, or B, are controlled by genes which differ in their location on the chromosome from genes controlling polysaccharide K antigens associated with most other O antigens.

Key words *Escherichia coli* genetic mapping K1 antigen (L) K4 antigen (L)

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By means of the bacterial agglutination technique the K antigens of *E coli* were originally described as L, A or B antigens. This division was based on the influence which heating of the culture to 100 C had on the agglutination reaction, the immunogenicity and the ability to bind antibodies (6)

During the years the difficulties in distinguishing between L and B antigens according to these criteria have become apparent and it has been realized that an examination of *E. coli* K antigens by newer serological methods, by chemical investigations and by genetical analyses is required both from a theoretical and a practical point of view The evaluation of K antigens has become of increased importance in the clinic during the last years.

The K antigens of *E. coli* have been numbered consecutively up to about 100 irrespective of the L, A or B variety The letter L, A or B has been added in a parenthesis after the K number Only about 70 can be demonstrated to-day as K antigens, while most of the remaining ones probably are non-existing

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(10) Their establishment was based on facts which to-day are worthless as evidence of presence of K antigens. In a previous communication it was shown that the genes controlling synthesis of two *E. coli* polysaccharide K antigens, K10 and K54 were genetically linked to the *ser A* locus and the name *kps A* was proposed for the K antigen controlling gene (13) K54 is a test K antigen, described as an L antigen while the K10 antigen examined is not the test K10 antigen (L) but the K antigen of the WG4 strain.

Many of the K antigen carrying *E coli* strains do not behave directly as suitable genetic recipients in crosses with existing *E coli* donor strains successful mating experiments were carried out with *K1(L)* and K4(L) strains as donors and described here

Furthermore, crosses have been carried out between a donor strain with K antigen mapped near the *ser A* locus and recipient strains *with O antigen O8 or O9 in order to examine* if donor and recipient K antigens were allelic.

These crosses were carried out because O8 and O9 strains are somewhat different from strains with other O antigens with respect to possession of K antigens. Among our classical K test strains we have both L, A and B labelled K antigens together with O antigens O8 and O9 No A antigens have been found in strains with O antigens other than O8, O9 and O101 The structural genes of the A antigens have not been mapped on the chromosome, but a *his* linked gene and perhaps one close to *trp* are believed to be involved (13 14) in the synthesis of A antigens. If this is so the genes determing these A antigens should be non allelic with those linked to *ser A* determining polysaccharide L antigens. As recipient strains the O8.*K27* (A) the O8.K8(L) and the O9.K57(B) strains were used.

MATERIALS AND METHODS

Organisms. The characteristics of the strains used are presented in Table 1 The K1 and K4 strains

TABLE 1 *Characteristics of Donor and Recipient Strains*

Wild-type strain	Designation of mutant	Mating type	Relevant characteristics	O K serotype
K12	Hfr P4X6	Donor	Met Lac$^+$	Rough:K
O1* = U5/41	D702 Lac	Recipient	Lac	O1:K1(L)
	D1620	Recipient	Pro His Srl$^+$ Mel$^+$ Str^r Nal	O1:K1(L)
	D1624	*Donor*	Trp Srl$^-$ Mel$^-$	*O1.K1(L)*
	D1626	Donor	His Xyl Mel Srl	O1:K1(L)
Test K4§ = U1/41	K4 Lac	Recipient	Lac	O5:K4(L)
	10/140	Donor	Trp	O5:K4(L)
	66/140	Donor	His	O5 K4(L)
Test K8 = G3404/41	*D1638*	*Recipient*	*Ser*	O8.*K8*(L)
WG4	D939	Donor	Trp Str^r Lac$^+$	O25:K10(L)
	D1114	Donor	Trp Str^r Nal^r	O25.K10(L)
Test K27 = E56b	D498	Recipient	Pro Mel$^-$ Xyl	O8.K27(A)
Test K54 = A12b	D1557	Recipient	Pro Ser Mel Xyl Srl$^+$ Str^r Nal^r	O6.K54(L)
Test K57 = H509d	D1643	Recipient	Ser Srl	O9:K57(B)

*Test strain of O antigen O1 §Test strain of K antigen K4
Met = methionine Pro = proline His = histidine, Trp = tryptophan Ser = serine Lac = lactose Srl = sorbitol, Xyl = xylose Mel = melibiose Str^r = streptomycin resistant, Nal = nalidixic acid resistant.
The Ser markers are *ser A* the Nal markers *nal A*.

(O1.K1.H7 and O5.K4.H4) were converted into donor strains by the method of F linked terminal selection (4, 5 8) using *E. coli* Hfr P4X6 and D939 as donors, as described previously (13)

Mutagenic agents. An auxotrophic or a carbohydrate utilization marker was introduced with N-methyl-N'-nitro-N-nitrosoguanidine by the method of *Adelberg et al.* (1) Mutants resistant to streptomycin or nalidixic acid were selected as spontaneous mutants on solid medium containing 100 μg or 25 μg/ml medium, respectively of these antibiotics.

Media. The minimal medium used (7) contained eosine methylene blue and lactose (EM-lac) or succinate plus galactose (EMS-gal) The complete media used were ox heart agar (pH 7 4) and bromothymol blue agar (pH 7 75) as used t Statens Seruminstitut. Ox heart agar (1 1 per cent agar) was prepared from 500 g ox heart meat, 10 g orthana peptone, 3 g NaCl and 2 g Na_2HPO_4, $12H_2O$ per litre tap water Bromothymole blue plates (1 per cent agar) were made of "broth for blue plates" containing per litre broth 1.5 ml metanol 5 per cent, 3 ml sodium thiosulphate 50 per cent, 15 ml bromothymol blue 1 per cent, 40.3 ml lactose 33 per cent and 1.8 ml glucose 33 per cent. "Broth for blue plates" was prepared from 15 g orthana peptone, 7.5 g NaCl and 7.5 g yeast extract per litre ionexchange water Fermentation characters were scored on bromothymol blue plates supplemented with appropriate carbohydrate (1 per cent) The complete medium used for the second purification of recombinants was eosine methylene blue agar (7)

Mating procedure 1 ml log phase cultures of both donor and recipient were filtered together through a Millipore filter (0.45 μm) The filter was transferred to the surface of a prewarmed broth agar plate incubated at 37° C for 2 hours and then placed in a flask containing 10 ml saline. By heavy shaking the bacteria were removed from the filter 0.1 ml samples of this "mating mixture and of 10^{-1} 10^{-2} and 10^{-3} dilutions of it were plated on selectiv media. Each parent was treated separately in the same manner as controls. After incubation for 24 to 72 hours at 37 C, recombinant clones were purified once on the same medium as used for their selection followed by one or two isolations on complete non-selectiv medium.

Antigenic analysis of recombinants. The presence of O and K antigens was scored by the agglutination test and by immunoelectrophoresis, as previously described (13) In the crosses, where most of the recombinants each contained tw different K antigens, the K antisera used for slide agglutination had to be diluted 1:2 instead of as usual 1:5 Several cases were in addition examined in double diffusion in gel as follow After 24 hours incubation t 37 C of a plat culture the plate was heated in an oven at 60° C for 1 hour Filter paper discs soaked with culture from these plates, together with paper discs soaked with antisera, were placed on a mm layer of "Reinagar" (Behringwerke) in a 14 cm plastic Petri dish in an appropriate pattern. The Petri dishes were stored in plastic bags for 24 hours at 37 C, read and kept at room temperature for another 24 hours before the final inspection was made. In the method previously described (15) the paper discs were soaked with extracts instead of plate culture.

Test for donor ability Sensitivity to phage MS2 was tested by the cross streaking method (2) In the case of the K1 containing strain agglutination in an f antiserum was used (11) as indication of acquirement of the donor ability together with a positive early reading of the test for sensitivity to phage MS2

RESULTS

Derivation of Donor Strains of Serotype O1 K1 and O5 K4

In order to obtain a K1 donor a lac⁻ derivative of the O1.K1 strain was mated with donor D939 Lac⁺ recombinants were scored for sensitivity to phage MS2 by the cross streaking method and, in this case, the result had to be read after only a few hours incubation of the plate in order to be interpreted properly Before this was understood, a slide agglutination test in an f antiserum was carried out, culture from some agglutinating and some non-agglutinating colonies was crossed with a K12 F strain and the donor state of the agglutinating colonies confirmed.

A donor strain of the O5.K4 culture was obtained from a cross with Hfr P4X6. Sensitivity to phage MS2 was distinct in this case.

Using the same technique of F linked terminal selection, no donor strains were obtained from O8.K8 and O9.K57 strains.

Transfer of K1 Antigen

Since we were interested to examine whether the genes for determination of K antigens, K1 and K4 were localized close to *ser A* at position 52 on the K12 linkage map (16) we concentrated, besides on *ser A* on the following markers which were easy to obtain *str A* (resistance to streptomycin) at position 64 *srl* (utilization of sorbitol) at position 51 *nal A* or *B* (resistance to nalidixic

TABLE 2. *Inheritance of Unselected Donor Markers among 74 Pro Recombinants from a Cross between Donor D1624 (O1 K1 Trp) and Recipient D1620 (O1 K1 Pro His)*

Number of recombinants	Marker inheritance						
	Pro	Mel⁻	Str^s	K1	Srl	Nal^s	His
74	74	2	44[a]	33[c]	36[b]	32[e]	65[d]

Tryptophan contraselection was employed against the donor parent.
33/44 (73 per cent) K1 [b] 30/36 (83 per cent) K1 29/32 (91 per cent): K1 [d] 33/65 (50 per cent) K1 33/33 (100 per cent) Str^s 30/33 (91 per cent) Srl⁺ 29/33 (88 per cent): Nal^s 33/33 (100 per cent): His

acid) at position 51 and 42, *his* at position 39 and *trp* at position 27 Mutants resistant to nalidixic acid, which had been isolated on medium containing 25 μg nalidixic acid per ml, turned out to be resistant to 40 μg/ml or more. Therefore it was considered most likely that the Nal marker was *nal A* (8) The donors chosen were *trp* or *his*⁻

The result of an intrastrain cross between the K1 donor strain D1624 and a *his*⁻ K derivative of the K1 strain line, D1620 is seen in Table 2. The result indicates that the K1 marker is situated between *str A* and *nal A*.

Another mating experiment was carried out between the K1 donor strain D1626 and a strain of serotype O6.K54 D1557 (Table) In this cross transfer of K1 was linked transfer of *ser A* irrespective of whether his was the selected or non-selected marker All recombinants which had received the K1 antigen had lost the K54 antigen. No transfer of the *nal A* marker was seen and only very few recombinants had become sorbitol negative.

In this cross recombinants were obtained at a frequency of 1 per 10⁸ donor cells and in the intrastrain cross D1624 × D1620 presented above the frequency was only 10 times higher The efficiency of transfer was of similarly low magnitude in the remaining crosses presented, in no case being higher than 1 recombinant per 10⁹ donor cells.

Transfer of K4 Antigen

In matings involving transfer of K4 antigen the Pro *ser A* O6.K54 recipient strain was crossed both with a *trp*⁻ (Table 4) and a *his* K4 donor strain. In both crosses a high percentage of the *ser A* recombinants were K4 again irrespective of whether *ser A* was the selected or non-selected marker All recombinants with K4 antigen had lost the K54 antigen.

TABLE 3 *Inheritance of Unselected Donor Marker by the Progeny of Cross between Donor D1626 (O1 K1 His) and Recipient D1557 (O6 K54 Pro Ser A)**

Selected marker	Number of recombinants	Marker inheritance								
		Pro	Mal	Xyl	Str^s	Ser	K1	Srl^s	Nal^s	O1
Ser A	98	1	1	27	33[b]	98[a]	83[d]	3	0	0
Pro	98	98	0	26[c]	39[f]	98[g]	78[h]	5	0	0

Histidine contraselection was employed against the donor parent.
25/27 (93 per cent) K1 [b] 27/33 (82 per cent) K1 83/98 (85 per cent): K1 [d] 25/83 (30 per cent): Xyl 27/83 (33 per cent) Str^s 83/83 (100 per cent) Ser⁺ 17/26 (65 per cent): K1 [f] 32/39 (82 per cent) K1 [g] 78/98 (80 per cent) K1 [h] 18/78 (23 per cent) Xyl 32/78 (41 per cent) Str^s 78/78 (100 per cent) Ser⁺

TABLE 4 *Inheritance of Unselected Donor Markers by the Progeny of a Cross between Donor 10/140 (O5 K4 Trp) and Recipient D1557 (O6+ K54 Pro Ser A)**

Selected marker	Number of recombinants	Marker inheritance Pro	Str[s]	Ser[+]	K4	Nal[s]	O5
Ser A	93	3	44[a]	93[b]	81	7	0
Pro[+]	55	55	25[d]	21	19[f]	0	0

*Tryptophan contraselection was employed against the donor parent.
40/44 (91 per cent) K4 [b] 81/93 (87 per cent) K4 40/81 (49 per cent): Str[s] 81/81 (100 per cent): Ser[+] [d] 15/25 (60 per cent) K4 18/21 (85 per cent) K4 [f] 15/19 (79 per cent) Str[s] 18/18 (95 per cent): Ser[+]

Examination of Allelism between ser A Linked K Antigens and K Antigen Associated with O8 and O9

If the area of the chromosome close to *ser A* controls the structure of the L antigens, K1 K4 K10 and K54 and if the assumption that the synthesis of a K antigen of the A variety *e.g.* K27 is located at another area (*his trp*) then they are non-allelic and it should be possible to create recombinants with two K antigens, *i.e.* one of the four L antigens mentioned and K27 No *ser A* K27 recipient existed but it was thought possible that transfer of *e.g.* K4 to a Met- K27 strain would take place in a few percentages of the Met+ recombinants. In a cross between the O5.K4 donor strain 66/140 and the O8.K27 recipient strain D498 a few recombinants were found to agglutinate on the slide in both K4 and K27 antisera in one recombinant tested by immunoelectrophoresis the presence of both K antigens was confirmed. No recombinants had received the K4 antigen and lost K27

In order to make similar experiments for allelism with recipients having O8 or O9 but no K antigens of the A variety the O8.K8(L) and O9.K57(B) strains were chosen as these were known to be fertile in crosses as recipients. The donor strain used was D1114 having K10 antigen. This strain was mated with the O9.K57 Ser- strain D1643 and the O8.K8 Ser- strain D1638. The outcome is presented in Table 5

In both crosses linkage was found between K antigen of donor and the selected Ser marker From this result it is concluded that the Ser marker in both recipient strains is *ser A* All recombinants with donor K antigen, except two, had kept K antigen of recipient, *i.e.* they have two K antigens. The two exceptions (cross D1114 × D1643) were of serotype O25.K10 *i.e.* both O9 and K57 antigens of recipient were lost. In cases where

TABLE 5 *Inheritance of Unselected Donor Marker among Ser Recombinants from Crosses between Donor D1114 (O25 K10 Trp) and D1643 (O9+ K57+ Ser) or D1638 (O8+ K8+ Ser) as Recipients**

Recipient	Number of recombinants	Marker inheritance Str[r]	Ser[+]	K10+	Str[s]	Nal[s]	O25
D1643	104	11	104	95[a]	38	9	3
D1638	105	1	103	62[b]	/	4	0

All were K57+ except two (see text)
[b] All were K8+
*Tryptophane contraselection was employed against the donor parent.

reading of the slide agglutination was doubtful the gel precipitation test was used to show presence or non-presence of K antigens.

Ten sorbitol positive recombinants from cross D1114 × 1643 having two K antigens were examined 1 month after the first examination for segregation of donor markers. None of about 600 colonies from each of the ten recombinants had lost the Srl⁺ trait. Twenty of these colonies from each recombinant were examined for the *ser A* marker for sensitivity to streptomycin and nalidixic acid and for presence of K antigens. No loss of donor markers was seen.

DISCUSSION

The most important aim of the recombination experiments reported here and previously (13) was to examine whether there is a genetical background for differentiation between the three kinds of *E. coli* polysaccharide K antigens, L, A and B as was originally done based on the bacterial agglutination method. The genes controlling enzymes concerned in synthesis of two L antigens, K10 and K54 were found previously to be located near *ser A*. In the present paper two additional K antigens, K1 and K4 originally described as L antigens, are examined and their mapping close to *ser A* is confirmed. Other L antigens should be examined, and it is to be anticipated that most of the so-called L antigens which in immunoelectrophoresis move away from the application basin towards the anode—in contrast to the A antigen which is also negatively charged but stays close to the application basin (10)—will be allelic to the four serological types examined so far. There will be exceptions, of course, and one, i.e. the L antigen K8 of the O8 K8 (L) strain, is reported here. This antigen was not lost when the L antigen K10 was acquired through recombination. The same applies to the K57 antigen of the O9 K57 strain and the K27 antigen of the O8 K27 strain. K57 has been described as a B antigen and K27 as an A antigen. From the cross D1114 (O25.K10) × D1643 (O9.K57) *ser A* the stability of the inherited donor markers of 10 recombinants was examined. No segregation was found. From this it is inferred that, in case the presence of two K antigens is due to a diploid state, this state is at least stable. In the cross previously reported (9) between an O25.K10 donor strain (W3703) and recipient strain O9.K26 *his* those recombinants which expressed the donor antigen O25 did not express recipient antigen K26 any more, indicating a location of the K26 genetical determinant near the *his* locus and the locus of the O antigen controlling gene (*rfb B*) (16).

In accordance with this we think that the reason for simultaneous presence of donor and recipient K antigens in the present studies is due to different chromosome locations of the K antigens.

To-day we have decided to abandon the distinction between L and B antigens. This decision is based on other examinations than bacterial agglutination (12) and we would like to denote all L antigens as K antigens. However the present results point at the possibility that the K antigens of O8 and O9 strains, whether labelled L, B or A antigens, are genetically mapped at loci different from other K antigens.

From a genetical point of view the following comment shall be made. The efficiency of transfer of the donor strains was low about 1 per 10^3 to 10 donor cells, as found previously (13). There are probably several reasons for this, such as restriction of foreign DNA deficient recombination enzymes, non-homology between the strains etc. A low efficiency of transfer was also found in the cross between the K1 and K1 derivatives of the same strain, U5/41. Thus, the donor strains which we obtained by the method of F linked terminal selection in crosses with the Hfr strain P4X6 (or D939) did not behave as true Hfr strains.

This fact, however is not considered of importance for the result obtained regarding the location of the gene(s) determining the structure of polysaccharide K antigens. An-

other method of obtaining donor strains will be attempted.

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THE TAXONOMY OF HAEMOPHILI ISOLATED FROM CONJUNCTIVAE

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The paper provides a description of 112 *Haemophilus* strains isolated from cases of conjunctivitis in Egypt, Tunisia, Denmark, and the U.S.A., and aims at a clarification of the taxonomy of haemophili implicated in conjunctivitis. Although the study confirms the diversity of haemophili which can be isolated from inflamed conjunctivae, the vast majority of strains could be assigned to either biotype II or III of *H influenzae* Thirteen strains possessed all the characteristics of the Koch-Weeks bacillus. However judged by the findings the recognition of the Koch-Weeks bacillus as a separate species does not seem tenable. It is suggested that this organism is considered a haemagglutinating variety of *H influenzae* biotype III The study indicates that the haemagglutinating ability *per se* which can be found in strains of several taxa of *Haemophilus* is associated with a special pathogenic potential.

Key words Haemophilus taxonomy conjuctivae isolation.

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Acute seasonal conjunctivitis of children especially in hot climates is a clinical entity of multiple etiology The common occurrence of mixed cultures in samples from conjunctivae during seasonal epidemics as well as during intervening periods makes it difficult to ascribe etiologic significance to specific species of microorganisms. However haemophili are among the bacteria most frequently isolated (22) and in particular the so-called Koch Weeks bacillus has long been incriminated as a cause of acute contagious conjunctivitis in certain parts of Europe, North Africa and South America. This organism was originally observed by *Koch* in 1883 (12) in Egyptian conjunctivitis exudates and first cultured by *Weeks* in New York city in 1886 (27) It is now usually known as *Haemophilus aegyptius* a name proposed by *Pittman & Davis* (20) They reported it to be distinguishable from *H influenzae* by serological means, to a certain extent also by growth characteristics, and by biochemical reactions such as differences in acid production from xylose and indole production, and by the presence of a haemagglutinin for human red blood cells (3) However the relation of the Koch-Weeks bacillus to *H influenzae* has remained the subject of much controversy The two groups of bacteria are closely related as revealed by

transformation studies (14 15) and in the recent 8th edition of *Bergey's Manual of Determinative Bacteriology* (1) *H aegyptius* is referred to as a *species incertae sedis*

Although it has been the custom, as pointed out by *Hart* (7) to label all haemophili from eyes as *H aegyptius* none of 120 conjunctivitis strains, isolated during an outbreak of seasonal conjunctivitis in Tunisia, were unambiguously identified as *H aegyptius*. This was also true for 114 strains isolated from conjunctivitis in England (8)

The present paper provides a description of 112 *Haemophilus* strains isolated from cases of conjunctivitis in Egypt, Tunisia, Denmark, and the U.S.A., and aims at a clarification of the taxonomy of haemophili implicated in conjunctivitis.

MATERIALS AND METHODS

Haemophilus strains. The origins of the 112 strains included in the study are as follows:

1) 25 strains isolated from sporadic cases of conjunctivitis at Danish hospital eye clinics during 1968–1973 with no known epidemiologic relationship.
2) 13 strains isolated during an outbreak of conjunctivitis in a Danish nursery school in 1972. Ten of the strains were isolated from eyes of young children (6–36 months old) and five strains from eyes of contacts.
3) 54 strains isolated from cases of seasonal conjunctivitis in Douz, Tunisia during January and February 1972, and August and September 1973 The geographical area and isolation techniques have been described previously (25, 26)
4) 7 strains isolated from cases of conjunctivitis in children from the Giza district south of Cairo, Egypt during November and December 1971 No seasonal outbreak of acute ophthalmia occurred during this period (6, 17)
5) 8 strains isolated from cases of seasonal conjunctivitis in Alexandria, Egypt. These strains were kindly provided by Professor *Hussein Hassan Faculty of Medicine Alexandria.*
6) 3 strains isolated from cases of conjunctivitis in Texas (20) *H aegyptius* strain NCTC 8502 (180a) suggested working type (23); *H aegyptius* strain NCTC 8134 (128a); *H aegyptius* strain NCTC 8135 (181a) These strains were obtained from the National Collection of Type Cultures, Colindale London.

Bacteriological methods. The methods used for cultivation of the bacteria, detection and typing of encapsulated strains, determination of haemagglutinating ability growth factor requirements, and biochemical activities have been described previously (9 10)

Determination of the nature of the X-factor The utilization of haemin or its precursor protoporphyrin IX as source of X factor was determined as described by *White & Granick* (29) using a proteose peptone medium (28) supplemented with either 6 × 10⁻⁶ M haemin (BDH) or 2 × 10⁻⁶ M protoporphyrin IX (Calbiochem, B grade) Bacterial growth in the media was estimated by turbidity measurements in a Spectronic 20 photometer (Bausch & Lomb) at 550 mμ.

RESULTS

Growth Factor Requirements

Of the 112 conjunctivitis strains studied, 104 required both X and V factors. The remaining eight strains required V factor only All of the 104 X-requiring strains, except for two, were capable of growing with protoporphyrin IX as supply of X factor *H aegyptius* strains NCTC 8134 and NCTC 8502 could not utilize protoporphyrin IX but required haemin for growth in the proteose peptone medium.

Forty two strains were haemagglutinating on primary testing, but several strains had lost the ability on subsequent examinations.

All X and V-requiring strains, except for four could be assigned to three of the biotypes of *H influenzae* (10) Four of the eight strains without X requirement belonged to *H parainfluenzae* whereas the remaining four strains constituted a separate unnamed taxon, tentatively labeled "Taxon A" in a previous paper (10) Each of the individual taxa will be described separately below The characteristics of the strains are shown in Table 1 Table 2 summarizes selected characters with special emphasis on the relationship of the strains to the Koch Weeks bacillus.

H influenzae biotype I Nine strains belonged to biotype I (Table 3) They were all non-encapsulated cocco-bacilli occasionally in filamentous form. Colonies on chocolate

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TABLE 1 *Biochemical and Physiological Characteristics of 112* Haemophilus *Strains Isolated from Cases of Conjunctivitis*

	No. of strains	V factor-requirement	ALA*→ Porphyrins	Indole	Urease	Ornithine decarboxylase	Lysine decarboxylase	Catalase	Glucose, acid	Sucrose acid	Lactose, acid	Xylose, acid	β-galactosidase (ONPG)	Haemagglutination	Nitrate reduction
H influenzae															
Biotype I	9	9	0	9	9	9	1	9	8	0	0	8	0	4	9
Biotype II	43	43	0	43	43	0	3	43	42	0	0	38	0	1	43
Biotype III	48	48	0	0	48	0	0	48	45	0	0	33	0	31	48
Unclassified	4	4	0	2	0	4	0	4	4	0	0	4	0	1	4
H parainfluenzae	4	4	4	0	2	4	0	4	4	4	0	0	1	2	4
Taxon A	4	4	4	4	0	4	4	4	4	4	0	3	0	3	4

* ALA δ-aminolaevulinic acid.

TABLE 2. *Summary of Selected Characters of 100 Classified X and V-dependent* Haemophilus *Strains Isolated from Cases of Conjunctivitis with Special Emphasis on Their Relationship to Koch-Weeks Bacillus*

BIOTYPE I
9 strains
(indole positive)

+ xylose —

8 | 1

+ haemagglutination — | no haemagglutination

4 | 4

BIOTYPE II
43 strains
(indole positive)

+ xylose —

38 | 5

+ haemagglutination — | no haemagglutination

1 | 37

BIOTYPE III
48 strains
(indole negative)

+ xylose —

33 | 15

+ haemagglutination — | + haemagglutination —

17 | 16 | 14 | 1

+ slender rods —

13 | 1

TABLE 3. *Relationship between Origins and Classification of 112 Haemophilus Strains Isolated from Individuals with Conjunctivitis*

	Total no. of strains	*H influenzae* biotype				*H parainfluenzae*	Taxon A
		I	II	III	UC*		
Sporadic cases							
Denmark	23	–	12	7(1)	1	3(1)	–
Outbreak in nursery							
Children	10	–	1	8(8)	1(1)	–	–
Contacts	5	–	5(1)	–	–	–	–
Dour, Tunisia							
(Endemic area)	56	4(1)	21	29(18)	2	–	–
Giza-district, Egypt							
(Endemic area)	7	–	2	–	–	1(1)	4(3)
Alexandria, Egypt							
(Endemic area)	8	5(3)	2	1(1)	–	–	–
Pittman strains							
Texas	3	–	–	3(3)	–	–	–

The figures in parentheses indicate the number of strains possessing a haemagglutinin.
UC Unclassified.

agar were smooth, convex, and grew to a diameter of 1–2 mm.

H influenzae biotype II The forty-three strains belonging to this biotype (Table 3) were cocco-bacilli. One of the strains isolated in Dour was capsulated and belonged to serotype b. Colonies on chocolate agar were smooth, convex, and reached a diameter of 1 2 mm.

H influenzae biotype III Forty-eight strains, including the Pittman strains NCTC 8134 NCTC 8135 and NCTC 8502, belonged to biotype III (Table 3) Most strains were cocco-bacilli, were non-encapsulated, and formed smooth, convex colonies reaching a diameter of ½–2 mm. Thirteen strains, including the three Pittman strains of *H aegyptius* were both xylose negative, haemagglutinating and were observed to be predominantly long slender rods (Table 2) These strains grew more feebly than other strains on all media. Of the 13 strains, nine were isolated in Dour, Tunisia, one in Alexandria, Egypt and three in Texas.

H influenzae unclassified The four unclassified X-dependent strains were cocco-bacilli. One of these strains from Denmark was encapsulated but did not react with antisera a-f

H parainfluenzae The four strains of *H parainfluenzae* (Table 3) belonged to biotypes I(2) and III(2) (10) The two strains of biotype III had haemagglutinating activity

Unnamed V-dependent strains. Four strains isolated in the Giza-district, Egypt formed a separate group. These strains have previously been described in detail under the preliminary label "Taxon A" (10) They were rods occasionally in filamentous form. Three of the strains were encapsulated and all four were spontaneously agglutinated by rabbit serum. Colonies on chocolate agar were smooth, convex and reached a diameter of 1–2 mm.

Epidemiology Table 3 provides a correlation between the origins of the 112 conjunctivitis strains and their classification. Eight of the ten strains isolated during the Danish nursery school outbreak had characteristics identical to *H influenzae* biotype III. However the child from whom the outbreak was first believed to have originated turned out to harbour a strain of biotype II The conjunctivitis of this child was clinically characterized as chronic.

Most of the Danish patients were examined clinically by one of us (C. H.M) at the time

of the bacteriological sampling. These included patients with acute as well as chronic conjunctivitis. The latter group had histories of chronic *Chlamydia trachomatis* infection, lacrymal duct obstruction, chronic dacryocystitis or herpetic keratitis. Table 4 shows the correlation between the clinical status of these patients and the haemagglutinating ability of the *Haemophilus* strains isolated from their eyes.

TABLE 4 *Relationship between Clinical Signs of Conjunctivae and the Haem gglutinating Ability of the Isolated* Haemophilus *St ains*

Haem-agglutination	Clinical status	
	Acute	Chronic
+	11	1
—	4	16

DISCUSSION

The present study confirms the diversity of *Haemophilus* strains which can be isolated from inflammed conjunctivae (2 7 8). This was evident for strains isolated in Denmark as well as in Tunisia and Egypt. However the vast majority of the 112 conjunctivitis strains studied could be assigned to either biotype II or III of *Haemophilus influenzae* (10). The latter biotype also included the three strains of *H aegyptius* isolated from conjunctivitis in Texas (20).

Davis et al. (3) found the haemagglutination test to be a valuable means of distinguishing *H aegyptius* from *H influenzae*. In the present study a haemagglutinin was demonstrated in strains belonging to all of the different taxa encountered. However a significant difference in the frequency of strains possessing the property was evident between the two predominant taxa. Only two per cent of the 45 strains of *H influenzae* biotype II had haemagglutinating activity compared to 65 per cent of the 48 strains of biotype III. Repeated examination showed that the property was easily lost.

Studies of a variety of bacterial species with haemagglutinating activity have revealed that the property is often associated with virulence and that fimbriae are likely to be the mediators of haemagglutination (4 5 13 18, 21 24). However the nature and significance of the haemagglutinin in certain strains of Haemophilus is not known. We have previously demonstrated that the property is an almost exclusive character of strains isolated from conjunctivae (10). The data presented here for the Danish strains moreover indicate that the capacity to agglutinate human red blood cells is associated particularly with strains from cases of acute conjunctivitis (Table 4). This affinity for human erythrocytes is likely to be a correlate of the ability of these bacteria to colonize healthy conjunctivae in significant numbers and thereby to cause acute infection. An analogous function has been suggested for fimbriae of *Moraxella bovis* involved in bovine conjunctivitis (19).

The non-haemagglutinating strains isolated from the eyes of patients with chronic conjunctivitis differed in no detectable way from the haemophili which form a part of the normal flora of the respiratory tract (10, 11). Many of these patients had in the past experienced a prolonged *Chlamydia trachomatis* infection, in some cases leading to a trachoma-like condition. Histories of pathologic conditions like obstruction of the lacrymal duct, chronic dacryocystitis and herpetic keratitis were also revealed. These conditions undoubtedly provide the basis for conjunctival colonization by endogenous bacteria, among them haemophili from the respiratory tract. Thus the presence of these bacteria is likely to be of secondary significance.

Thirteen of the 112 conjunctivitis strains studied possessed all the phenotypic traits of *H aegyptius* as defined by *Pittman & Davis* (20) (Table 2). These strains, which included the suggested working type of *H aegyptius* were isolated in Texas, Tunisia and Egypt. Among all the X and V-dependent strains, however there were examples of strains which possessed all combinations of the characteristics evaluated (Table 2).

White & Granick (29) in a study of the haemin biosynthesis in *Haemophilus* strains found that a strain of *H aegyptius* differed from strains of *H influenzae* in its lack of the enzyme (ferrochelatase) which inserts iron into protoporphyrin IX to convert it to haeme. This finding suggested a possible means of distinguishing *H aegyptius* from *H influenzae* but this was not supported by the present findings. In this study only two of the reference strains of *H aegyptius* showed a requirement for haemin *per se*. All other X-dependent strains, including the third reference strain of *H aegyptius* grew well utilizing the precursor protoporphyrin IX.

Judged by the findings of the present study the recognition of Koch Weeks bacillus as a separate species as proposed by *Pittman & Davis* (20) does not seem tenable. We prefer to consider such strains as haemagglutinating varieties of *H influenzae* biotype III. However this study suggests that the haemagglutinating ability *per se* which can be found in strains of several taxa of *Haemophilus* is associated with a special pathogenic potential.

Four strains isolated in the Giza-district in Egypt formed a separate taxon. The growth factor requirement and the DNA base composition of these strains (10) suggest a relationship to the *H parainfluenzae* group whereas several of their biochemical characteristics are similar to those of the *H influenzae* group. A spontaneous agglutination in normal rabbit serum, as seen in these strains, has previously been reported for strains of Koch-Weeks bacillus by *Luerssen* (16). With the small number of strains available, we do not feel justified in drawing any firm conclusions as to the precise allocation of these strains.

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IDENTIFICATION OF *NEISSERIA GONORRHOEAE* IN CULTURES FROM TONSILLO-PHARYNGEAL SPECIMENS BY MEANS OF A SLIDE CO-AGGLUTINATION TEST (PHADEBACT® GONOCOCCUS TEST)

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Menck, H. Identification of *Neisseria gonorrhoeae* in cultures from tonsillo-pharyngeal specimens by means of a slide co-agglutination test (Phadebact® Gonococcus Test). Acta path. microbiol. scand. Sect. B, 84 139–144 1976.

A slide co-agglutination test (Phadebact® Gonococcus Test) for the serological identification of *Neisseria gonorrhoeae* was assessed on gonococcal-like, oxidase positive colonies from 120 cultures, originating from about 6,500 consecutive tonsillo-pharyngeal specimens received at the Neisseria Department, Statens Seruminstitut. The test was performed after subculture on serum-free medium, since this procedure was found to reduce the number of strains showing inconclusive reactions (pseudo co-agglutination). If this pseudo co-agglutination does occur however, the test can be repeated with the addition of trypsin to the test system. This causes the previously inconclusive reactions to be reverted to clearly positive reactions in the case of gonococci, and to clearly negative reactions in more than half of the previously inconclusive reactions with other bacterial strains. The results obtained by the Phadebact Gonococcus Test were compared with those obtained by bacteriological identification procedures. Fifty-six of the 120 cultures examined contained gonococci, and all strains were identified by the slide co-agglutination test (five strains with the addition of trypsin). The remaining 64 cultures were negative or exhibited consistently pseudo co-agglutination (eight strains). The specificity and sensitivity of the reagent was further confirmed by the examination of 55 strains of *Neisseria gonorrhoeae* and 50 strains representing *Neisseria* species commonly occurring in tonsillo-pharyngeal specimens. The Phadebact Gonococcus Test was considered to be a reliable alternative to routine bacteriological identification of *Neisseria gonorrhoeae*.

Key words: *Neisseria gonorrhoeae*; identification; tonsillo-pharyngeal specimen; slide co-agglutination.

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The direct immunofluorescence test has proved to be a useful tool for identification of *Neisseria* (*N.*) *gonorrhoeae* in cultures from urogenital and rectal specimens (9). However, gonococci in cultures from pharyngeal specimens must still be confirmed by more time consuming bacteriological tests, mainly due to a certain staining of some strains of *N. meningitidis* in the direct im-

munofluorescence test (1 8, 14) The problem of specificity seems to be overcome in another method for rapid serological identification of *N gonorrhoeae* viz the slide co-agglutination test described by *Danielsson & Kronvall* (3) In that test, which was first developed for typing of pneumococci (7) serological grouping of streptococci (?) and mycobacterial typing (6) cells of protein A producing staphylococci (*Staphylococcus aureus* strain Cowan 1) are used as carrier particles for antibodies of known specificity

In the present study a commercially available co-agglutination reagent, claimed to be specific for *N gonorrhoeae* (Phadebact® Gonococcus Test), has been used for the identification of gonococci. One hundred and twenty cultures, representing about 6,500 consecutive tonsillo-pharyngeal specimens received at the Neisseria Department, Statens Seruminstitut, during a four month period were examined The results were compared with those obtained by routine bacteriological identification procedures.

MATERIALS AND METHODS

Media

Selective medium The HYL medium was prepared as described by *Reyn* (12) and *Møller & Reyn* (10) with one modification, *viz.* haemoglobin was replaced by defribinated horse blood (13) The following antibiotics were added Polymyxin B sulphate 25 IU/ml, nystatin 25 IU/ml and vancomycin 2 µg/ml.

Non-selective medium This was the same as the selective medium, except that the antibiotics were omitted. Trimethoprim 3 µg/ml was added to inhibit the swarming of Proteus.

Fermentation medium (SFF) A serum-free fermentation medium developed at the Media Department, Statens Seruminstitut, has also been used This consisted of 882 ml ion-exchanged tapwater 9 g peptone (Orthana) 3 g yeast extract (Oxoid) 3 g NaCl 3.84 g Na_2HPO $12H_2O$ 6 g starch (1252 Merck) The pH was adjusted to 7.2 with NaOH. Addition of 8.12 g Japan agar 4022 Autoclaved for 20 minutes at 120° C followed by the addition of 18 ml glutamin 2 per cent, 18 ml phenol red 1.500, 15 ml hæmin 0.2 per cent, 5.5 ml nystatin 4000 E/ml, 9 ml co-carboxylase 0 1 per cent, and 30 ml glucose 33 per cent. The pH was adjusted to 7 7 with NaOH The pH the next day was 6.5–8.4

Bacterial Strains

Fifty-three freshly isolated strains of *N gonorrhoeae* were obtained from the routine material. In addition, 28 strains of *N meningitidis* isolated from pharyngeal specimens, 9 of *N lactamica*, 1 of *N subflava* (ATCC 11075) 1 of *N flavescens* (ATCC 13120) 1 of *N perflava* (ATCC 10555) and 9 of *N catarrhalis* were used in the experiments.

Clinical Specimens

Tonsillo-pharyngeal specimens sent in on charcoal impregnated sterile wooden applicators in a modified Stuart medium (12) for identification of *N gonorrhoeae* at the Neisseria Department, Statens Seruminstitut, were inoculated on two chocolate agar media, one selective and one non-selective. After 18 to 24 hours of incubation at 36 C in a moist atmosphere containing about 10 per cent CO_2, the plates were examined by experienced technicians. All gonococcal-like, oxidase positive colonies were selected for further identification by Gram stained smears, fermentation tests, and the ability to grow on Dubos oleic acid agar medium (4) In the present study identification was obtained for all Gram negative cocci or short, plump Gram negative rods, either at the Neisseria Department or at the Department of Diagnostic Bacteriology Statens Seruminstitut. The remaining Gram negative rods were not examined further

Subcultures were made simultaneously on SFF medium. After 18 to 23 hours of incubation, these cultures were used for the slide co-agglutination test. Cultures were not left for more than five hours at room temperature before the test was performed.

Performance of the Slide Co-agglutination Test

The gonococcal (batch 1011) and control (batch 1012) reagents were obtained from Pharmacia Diagnostic AB. The enclosed directions for use of the reagents were followed. Two samples of the suspected culture were smeared out on a glass slide without any addition of liquid. One drop of specific gonococcal reagent and one drop of control reagent were each mixed well with the smears by means of a platinum loop (gauge 0 8 mm) Some experience, especially as regards the proper amount of bacterial material in relation to the amount of reagent, was necessary in order to obtain clear-cut results. The glass slide was rocked gently and then read against a dark background in oblique trans-illumination. Co-agglutination occurred within one half to one minute and was recorded either as positive co-agglutination, negative co-agglutination or as pseudo co-agglutination. No grading of the positive co-agglutination reactions was used, since almost all could be described

as ++ reactions when the grading system and photographs published by *Danielsson & Kronvall* (3) were used as reference.

In this context, the term pseudo co-agglutination covered any agglutination that took place with both the specific gonococcal reagent and control reagent (see Fig. 1). When pseudo co-agglutination occurred, the test was repeated, but with one modification, viz. one drop of distilled water containing 1 mg trypsin/ml was applied to the dried smears prior to the drop of reagent.

TABLE 1 *Examination of 53 Strains of* N gonorrhoeae *by the Slide Co-agglutination Test Performed after Growth on Selective or Non-selective Media*

Slide co-agglutination test	Selective medium	Non-selective medium
Positive co-agglutination	27	23
Negative co-agglutination	0	0
Pseudo co-agglutination	26	30
Total	53	53

inconclusive reaction.

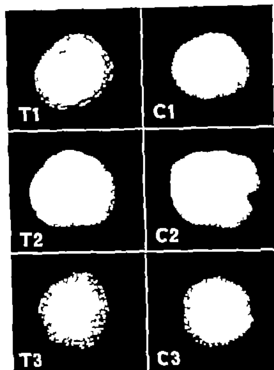


Fig 1 Identification of *N gonorrhoeae* by means of Phadebact® Gonococcus Test. Co-agglutination of *N gonorrhoeae* by Phadebact gonococcus reagent (T1) and the control reagent (C1). T2 and C2 show the reactions of the same reagents with a strain of *N meningitidis*. By way of comparison, an inconclusive pseudo co-agglutinating strain of *N catarrhalis* (T3 and C3) is shown.

RESULTS

Preliminary Experiments

In preliminary experiments, 53 strains of *N gonorrhoeae* were subjected to the slide co-agglutination test after subculture on selective and non-selective medium, both used in the routine primary isolation of *N gonorrhoeae*. As shown in Table 1 approximately 50 per cent of the strains gave pseudo co-agglutination in either case. These reactions, whether obtained with the specific reagent or with the control reagent, often resembled that produced by the gonococcal reagent in a typical positive co-agglutination reaction (see T1 Fig. 1). For that reason, the test was repeated after subculture on the less complex SFF medium. The number of pseudo co-agglutinating strains was then reduced considerably. Therefore, in the present study the slide co-agglutination tests were made on the strains isolated after subculture on SFF medium. It should be noted that the pseudo co-agglutination reactions still found after subculture on the SFF medium did not look like those which were found with the gonococcal reagent in positive co-agglutination. However they might resemble those found in a strongly positive co-agglutination reaction (++++ co-agglutination according to *Danielsson & Kronvall* (3)) (see T3 Fig. 1). In addition, the pseudo co-agglutination occurred more rapidly in contrast to a true co-agglutination.

The results of the preliminary investigations also indicated that the period of time (<5 hours) the cultures were left on the bench at room temperature was not responsible for the occurrence of pseudo co-agglutination.

In order to test the specificity of the reagent, 50 *Neisseria* strains, mostly *N meningitidis* were subjected to the slide co-agglutina-

tion test. All gave negative co-agglutination with or without trypsin, except one strain of *N lactamicus* which remained pseudo co-agglutinable with trypsin.

Clinical Material

The material studied comprised 120 cultures containing gonococcal like, oxidase positive colonies. The cultures represented about 6,500 consecutive tonsillo-pharyngeal specimens and originated from 120 different patients.

From routine and diagnostic procedures, the primarily suspected cultures were 56 strains of *N gonorrhoeae* 7 of *N meningitidis* 4 of *N lactamicus* 5 of *N catarrhalis* 2 of *Moraxella osloensis* plus 46 strains of Gram negative rods for which no identification was obtained. (See Table 2)

The results of the co-agglutination test are summarized in Table 2. Positive co-agglutination was obtained only with strains of *N gonorrhoeae* (51 strains) and none of the strains of *N gonorrhoeae* gave negative co-agglutination. Only 5 of the strains of *N gonorrhoeae* gave pseudo co-agglutination. Forty-five of the other strains gave clearly negative co-agglutination and 19 gave pseudo co-agglutination. Altogether 24 (20 per cent) of the gonococcal-like oxidase positive strains gave pseudo co-agglutination.

Table 3 shows that all *N gonorrhoeae* strains primarily showing pseudo co-agglutination had reverted to clearly positive co-agglutination reactions with trypsin. Eleven of the other *strains had reverted to* clearly negative reactions. For 8 (33 per cent) of the total number of pseudo co-agglutinating strains or 7 per cent of the total number of gonococcal-like, oxidase positive strains, it was not possible to obtain conclusive results.

DISCUSSION

A reagent for the serological identification of *N gonorrhoeae* originally developed by *Danielsson & Kronvall* (3) is available commercially as Phadebact® Gonococcus Test.

Gonococcal cultures obtained from the selective and non-selective media used at the Neisseria Department for primary isolation of *N gonorrhoeae* were found to be unsuitable for the slide co-agglutination test, since approximately half of the strains agglutinated antibody coated staphylococcal cells as well as control cells (pseudo co-agglutination). Since non-specific reactions might be caused by inter-action between the protein A of the control cells and horse serum globulins from

ABLE 2. *Slid Co-agglutination Test Performed on 120 Cultures Contain g Gonococcal-like Oxidase Positive Colonies (without the Addition f Trypsin t the Test System)*

Bacterial strains		Slide co-agglutination test		
Species	Number	Positive co-agglutination	Negative co-agglutination	Pseudo co-agglutination
N meningitidis	7	0	5	2
N lactamicus	4	0	1	3
N catarrhalis	5	0	1	4
Moraxella osloensis	2	0	1	1
Gram negative rods	46	0	37	9
Total	64	0	45	19
N gonorrhoeae	56	51	0	5
Total	120	51	45	24

Inconclusive reaction.

TABLE 3 *Slide Co-agglutination Test Repeated with the Addition of Trypsin to the Pseudo-Co-agglutinating Strains*

Pseudo co-agglutinating strains		Slide co-agglutination test + trypsin		
Species	Number	Positive co-agglutination	Negative co-agglutination	Pseudo co-agglutination*
N meningitidis		0	2	0
N lactamicus	3	0	0	3
N catarrhalis	4	0	3	1
Moraxella osloensis	1	0	1	0
Gram negative rods	9	0	5	4
Total	19	0	11	8
N gonorrhoeae	5	5	0	0
Total	24	5	11	8

* Inconclusive reaction.

the medium contaminating the bacterial suspension, the strains were retested after subculture on serum-free medium (see materials and methods) After growth on this medium the majority of gonococcal strains gave clear cut positive reactions and none was negative. In the case of persisting pseudo co-agglutination, the test was repeated with trypsin added to the test system. Gonococci could then be identified by the slide co-agglutination test in all out of 56 cultures from throat swabs (see Tables 2 and 3) in which the routine bacteriological methods had revealed the presence of *N gonorrhoeae*

Subculture on a serum-free medium delays diagnosis by one day However the growth of *N gonorrhoeae* is often sparse and the culture contaminated on the primary plate from tonsillo-pharyngeal swabs. Thus, subculture would often be necessary also before bacteriological identification can be initiated.

The serum-free medium used in this work, SFF medium, seemed to be less suitable for growth of gonococci than the routine selective medium. In a small experiment, 68 strains of *N gonorrhoeae* were subcultured on the SFF medium with and without antibiotics (the same as in selective media) with the selective medium serving as control. All 68 strains were recovered from all three media, but it applies to 89 per cent of the strains grown on SFF medium without antibiotics, and to 50 per cent of the strains grown on SFF medium with antibiotics, that growth was less abundant than on the control medium.

Cultures obtained from the following commercially available media for primary isolation of *N gonorrhoeae* might be suitable for the slide co-agglutination test Bacto GC medium containing haemoglobin and yeast supplement B (Difco laboratories) BBL chocolate medium with "IsoVitaleX" supplement (Baltimore Biological Laboratories) and haemoglobin-free Thayer Martin medium (5 11) *In a small supplementary experiment* 65 strains of *N gonorrhoeae* were subjected to the slide co-agglutination test after subculture on *Bacto GC medium containing* haemoglobin and yeast supplement B. Only 6 out of the 65 strains showed pseudo co-agglutination *and 4 of these* were reverted to clearly positive co-agglutination reactions after the addition of trypsin.

During the four-month period in which the present experiment was carried out, 98,460 specimens were received from all parts *of Denmark with a view to identification of N gonorrhoeae* About 6,500 were tonsillo-pharyngeal specimens. Of these, 120 cultures contained gonococcal-like oxidase positive

colonies, 56 of which proved to be *N gonorrhoeae* The results obtained by the slide co-agglutination test were in agreement with those obtained by bacteriological procedures. For 8 out of 120 strains, which did not include any strains of *N gonorrhoeae* it was not possible to reach any conclusive result. The examination of Gram stained smears from these cultures would exclude some strains from further examination. As regards the remaining 4 cultures consisting of Gram negative diplococci or short, plump Gram negative rods, it was necessary to identify the strains bacteriologically

The Phadebact Gonococcus Test reagent was found to have high specificity and sensitivity being equally apparent from examinations of 103 known strains belonging to the *Neisseriaceae* and examinations of 120 unknown strains obtained from tonsillo-pharyngeal specimens received for routine diagnosis of *N gonorrhoeae* It might therefore be expected that the results obtained by the slide co-agglutination test would be in concordance with those obtained by routine procedures performed on gonococcal like oxidase positive colonies isolated from urethral, cervical, and rectal specimens. The direct immunofluorescence technique can be used to identify *N gonorrhoeae* from these localities. It is an adequate and rapid method (9) but in smaller laboratories where the direct immunofluorescence technique cannot be used owing to the lack of suitable equipment, conjugate etc, the slide co-agglutination test would be an acceptable alternative to fermentation tests and other bacteriological procedures.

The Phadebact® Gonococcus Test reagents were kindly supplied by *Pharmacia Diagnostic AB* Uppsala, Sweden. I wish to thank Dr *Inga Lind* for her advice and assistance in preparation of this manuscript.

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Acta path. microbiol. scand. Sect. B, 84 145–153 1976

STREPTOCOCCAL BACTERIOPHAGE 12/12 BORNE HYALURONIDASE AND ITS CHARACTERIZATION AS A LYASE (EC 4.2.99.1) BY MEANS OF STREPTOCOCCAL HYALURONIC ACID AND PURIFIED BACTERIOPHAGE SUSPENSIONS

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Niemann, H., Birch-Andersen, A., Kjems, E., Mansa, B. & Stirm, S. Streptococcal bacteriophage 12/12-borne hyaluronidase and its characterization as a lyase (EC 4.2.99.1) by means of streptococcal hyaluronic acid and purified bacteriophage suspensions. Acta path. microbiol. scand. Sect. B, 84 145–153 1976

Hyaluronic acid was obtained from filtrates of heat killed cultures of *Streptococcus pyogenes* group A, strain K56, by simple ethanol precipitation and treatment with an adsorbent. The hyaluronic acid is pure as judged from chemical and sedimentation analyses. Particles of streptococcal bacteriophage 12/12 were isolated from phage-lysed group A streptococci by polyethylene glycol precipitation and isopycnic centrifugation. Electron micrographs of negatively stained preparations showed a typical Bradley group B virus with a long, flexible, cross-striated tail and a knob- or star-like structure at the distal tip of the tail. The hyaluronic acid is depolymerized upon incubation with the phage 12/12 virions. After extensive digestion, a mixture of at least four oligosaccharides is formed, the two smallest of which are a tetra- and an octasaccharide terminating in reducing *N*-acetyl-D-glucosamine. The tetrasaccharide shows an absorption maximum at 231.5 nm with a molar extinction coefficient = 4820 litres × mole × cm^{-1} and it is therefore concluded that the bacteriophage-borne hyaluronidase catalyses a β-elimination. Accordingly it is classified as hyaluronate lyase (EC 4.2.99.1)

Key words: Streptococcal bacteriophage 12/12-borne hyaluronidase; lyase.

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From lysogenic haemolytic group A streptococci, bacteriophages can be isolated which are capable of infecting certain strains of streptococci. In contrast to virulent phages these lysogenic phages can infect encapsulated group A streptococci (7 8, 9 10 12). During reproduction, these viruses induce the formation of hyaluronidases which belong to at least four serological groups (9 11 12) all of which are serologically unrelated to streptococcal hyaluronidase. It was shown (9) that

colonies, 56 of which proved to be *N gonorrhoeae* The results obtained by the slide co-agglutination test were in agreement with those obtained by bacteriological procedures. For 8 out of 120 strains, which did not include any strains of *N gonorrhoeae* it was not possible to reach any conclusive result. The examination of Gram stained smears from these cultures would exclude some strains from further examination. As regards the remaining 4 cultures consisting of Gram negative diplococci or short, plump Gram negative rods, it was necessary to identify the strains bacteriologically

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Fig. 2 A. Electron micrograph of streptococcal bacteriophage 12/1 purified by CsCl density gradient centrifugation. Note the absence of impurities and the hollow tail of a particle with an empty head (arrow). Negatively stained with ammonium molybdate. The bar on each electron micrograph represents 100 nm. 180,000 ×

Fig. 2 B. Electron micrograph of a phage 12/12 particle purified as for Fig. 2 A, but negatively stained with uranyl acetate. The knob at the tail end is well illustrated. Note part of a hollow tail at bottom of picture. 280,000 ×

12/12 lysates by addition of 6.5 per cent or more of polyethylene glycol 6,000 analogous to earlier results (24). On isopycnic centrifugation of concentrated virus suspensions the phages banded at 1.43 g/ml, below all contaminants. Electron microscopy of the phages in this band showed that the virus particles appeared practically free of impurities (Fig. 2 A and 2 B). Aliquots of the same material were tested for hyaluronidase activity and 1 TRU per about 4 × 10⁹ PFU was found to be present. No hyaluronidase activity was detected in the lighter fractions obtained by isopycnic centrifugation, except in a band around 1.20 g/ml. Electron microscopy of material from this band showed many loose phage tails present with practically no phage heads to be seen (Fig. 3 A and 3 B).

Previously published electron micrographs of shadow cast phage 12/12 particles revealed isometric heads of about 480–500 Å diameter and long flexible cross-striated tails approximately 1700 Å long and 70–100 Å thick (9, 12). These observations have been confirmed on the negatively stained phage particles of the present material (Fig. 2 A and 2 B). Furthermore in this material some of the particles carry a knob- or star like structure at the end of the tail (Fig. 2 B). Many of the virus particles are seen to have lost their nucleic acid (empty heads, Fig. 2 A). On such particles the tails appear hollow as heavy

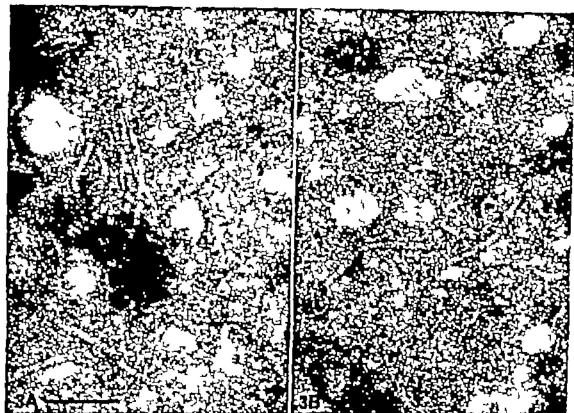


Fig 3 A and 3 B. Electron micrographs of material from the second band from the top after a discontinuous CsCl density gradient centrifugation ($\rho = 1.20$ g/ml) Free phage tails are the only morphologically recognizable structures Some unidentifiable debris is also present. White background patches represent repellant regions with no stain on the supporting film and are thus artifacts. Negatively stained with ammonium molybdate. 180 000 ×

metal stain is seen to have penetrated the tail cores (Fig 2 A and 2 B)

Antibody Formation by Immunization with Phage Particles and Phage Tails

The purified phages and free phage tails induced the formation of antibodies against plaque formation as well as of hyaluronidase neutralizing antibodies. Thus, the sera are qualitatively similar However the levels of antibodies of both specificities were much higher in the sera from the rabbits inoculated with intact phages.

Depolymerization of Isolated Hyaluronic Acid by Purified Phage 12/12 Particles

Incubation of a solution of the purified bacterial hyaluronic acid with purified phage particles (about 10^{10} PFU/ml) at 37 C gave a rapid loss of viscosity and a simultaneous increase in reducing ability Fig 4 gives the results of a representative experiment. No loss of viscosity was observed even after 18 hours of incubation at 37 C in a control experiment omitting only the phage particles.

The depolymerization products obtained after prolonged (18 hours at 37 C) incubation with the virus particles were purified by gel filtration and then separated by ion exchange chromatography All hexuronic acid was eluted in the separation volume of the Sephadex G100 column. Four peaks (A-D) were obtained in the ion exchange chromatogram by elution with a linear NaCl gradient from a DEAE Sephadex A25 column when fractions were tested for hexuronic acid (Fig 5) The retention of all hexuronic acid by

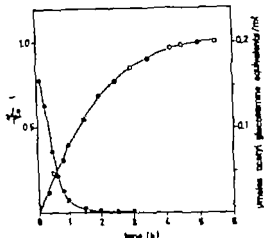


Fig. 4 Depolymerization of streptococcal hyaluronic acid by particles of bacteriophage 12/12. A 0.1 per cent (w/v) solution of hyaluronate in a 0.05 M TRIS/HCl buffer of pH 7.2, containing 10^{-} M Mg^{++} and 10^{-} M Ca^{++} was incubated at 37 C with 10^{10} PFU (= 2.4 TRU) of purified 12/12 viruses per ml. The loss of specific viscosity ($\frac{\eta}{\eta_0}-1$; —●—●—●—) and the increase in reducing ability (*N*-acetyl-D-glucosamine equivalents/ml —○—○—○—) is plotted versus the incubation time.

gel filtration together with the final yields of saccharides A to D (see Material and Methods) indicated that the incubation had resulted in a complete degradation of the hyaluronic acid to a mixture of at least four fragments. In two independent experiments relative amounts of about A/B/C/D = 1/1.2/2.3/1.25 were obtained.

To identify the reducing sugar and to estimate the size of the fragments, oligosaccharides A and B were analysed for hexuronic acid and glucosamine before and after reduction with sodium borohydride. The reduction had no effect on the hexuronic acid analysis with carbazole-sulfuric acid but an average of about 51 and 27 per cent of the glucosamine was reduced in A and B, respectively. Thus, oligosaccharide A is a tetrasaccharide and oligosaccharide B an octasaccharide.

The ultraviolet absorption spectrum of tetrasaccharide A at pH 7 showed a maximum at 231.5 nm indicating the presence of a double bond (13). The molar extinction coefficient was determined to ε = 4820 litres × $mole^{-1}$ × cm^{-1}.

DISCUSSION

Hyaluronidases from different sources depolymerize their common substrate at different sites, by different mechanisms and yield fragments of different sizes (13 15 23) (see also Fig 6). In this context, we investigated the mode of action of an enzyme induced by and—partially—associated with bacteriophage particles which can infect β-haemolytic group A streptococci (8 9 11 12). As a substrate we used streptococcal hyaluronic acid obtained from cultures of the purely mucoid phage detector strain K56 and as an enzyme streptococcal phage 12/12 particles concentrated and purified from the lysates by polyethylene glycol precipitation and isopycnic centrifugation.

Upon incubation of a solution of the streptococcal hyaluronic acid with purified phage 12/12 virions, a rapid loss of viscosity and a concomitant increase in reducing ability of the polysaccharide was observed (Fig. 4). After prolonged interaction a mixture of hyaluronic oligosaccharides was obtained from which four fractions, together accounting for 86.5 per cent of the starting material, could be purified by successive gel filtration and ion exchange chromatography (Fig 5). In the smallest fragments about one-half (fraction A) or one-fourth (fraction B) of the glucosamine, but no glucuronic acid, could be reduced with sodium borohydride, showing that A was a tetra and B an octasaccharide, and that N-acetyl-D-glucosamine was the reducing sugar. The UV spectrum of the tetrasaccharide A showed the same absorption maximum with the same molar extinction coefficient as reported by other authors (13) for the unsaturated hyalobiuronic acid with 4-deoxy-α-L-threo-hex-4-enuronic acid as the non reducing sugar.

In total, these findings led us to conclude that the phage 12/12 enzyme catalyses a β elimination which forms unsaturated hexuronic acid as the non-reducing, and N

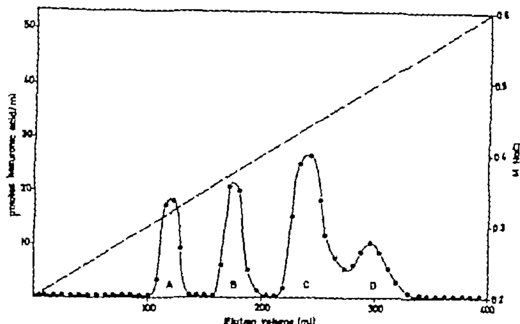


Fig 5 Ion exchange chromatography of hyaluronic oligosaccharides produced by bacteriophage 12/12 degradation. The mixture of depolymerization products obtained from 60 mg of hyaluronic acid by prolonged action of virus particles (18 hours at 37 C, compare Fig 4) was desalted and then applied to a DEAE Sephadex A25 column (20 cm length, 1 1 cm² cross-section area) from a 0.05 M TRIS/HCl buffer of pH 7.2. Elution was carried out at 18 ml/hour with a linear NaCl gradient (400 ml) from 0.2 to 0.6 M (— — — —) The fractions were tested for hexuronic acid with carbazole-sulfuric acid (—●—●—●—)

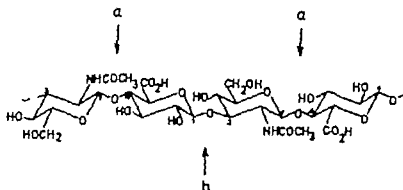


Fig 6 Diagram illustrating the cleavage of hyaluronic acid by different enzymes. Bacterial lyases and the lyase associated with particles of streptococcal bacteriophage 12/12 cleave at sites "a" by β-elimination. The testis and leech hydrolases, on the other hand, cleave by hydrolysis at sites "a" or "b" respectively

acetyl-glucosamine as the reducing end sugar (Fig. 6)

The electron micrographs of negatively stained specimens of streptococcal phage 12/12 (Fig. 2 A and 2 B) show virus particles with a morphology similar to bacteriophages belonging to Bradley group B (3) thus resembling the T1 T5 and lambda *E. coli* phages, *Salmonella typhi* Vi phage II etc Analogous to the spike associated glycanases

of some *E. coli* and *Klebsiella* bacteriophages (1 18, 19) the active centre of the 12/12 lyase may well be part of the knob- or star like structures present at the end of the tail. Our immunization experiments with suspensions of highly purified but intact phages and suspensions containing only free phage tails may also lend some support to this concept. The antisera obtained indicated that at least part of the hyaluronidase is phage tail associated.

We wish to express our gratitude to Miss Beate Bebrig for skilful technical assistance and to Miss *Helga Kochanowski* for the analytical ultracentrifugation.

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BIOLOGICAL CONDITIONS INFLUENCING THE FOCAL NECROTIC HEPATITIS TEST FOR DIFFERENTIATION BETWEEN HERPES SIMPLEX VIRUS TYPES 1 AND 2

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Mogensen, S. C. Biological conditions influencing the focal necrotic hepatitis test for differentiation between herpes simplex virus types 1 and 2. Acta path. microbiol. scand. Sect. B, 84: 154–158, 1976.

Some biological conditions of the focal necrotic hepatitis test for the differentiation between herpes simplex virus (HSV) types 1 and 2 were investigated. Most of 15 different strains of mice tested were found usable in the test. An upper age limit (4 weeks) for the appearance of focal necrotic liver lesions was found in one strain of mice, while this was not seen in another strain. The minimum dose in 3- to 4-week-old mice was found to be as small as 10^2 to 10^4 p.f.u. in 0.1 ml of diluent. Suckling rats and hamsters, aged up to 7 and 14 days, respectively, were found to be convenient as alternative test animals. Finally, it was observed that focal necrotic hepatitis did not develop in the nude mouse with thymic aplasia on intraperitoneal inoculation of HSV type 2. The possible involvement of the thymus in the pathogenesis of the focal necrotic lesions is briefly discussed.

Key words: Herpes simplex virus types 1 and 2; focal necrotic hepatitis test; differentiation.

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Strains of herpes simplex virus (HSV) are classified into two distinct groups, type 1 and type 2, on the basis of antigenic differences detectable by various serological procedures (*Plummer* 1964, *Pauls & Dowdle* 1967). In addition to antigenic differences, the two virus types show different clinical and epidemiological characteristics (*Nahmias & Roizman* 1973), and it has been investigated whether a number of biochemical and biological properties of HSV might be used for differentiation between the two virus types (*Nahmias & Dowdle* 1968).

In a previous paper (*Mogensen et al.* 1974) it was reported that HSV type 2 strains produce macroscopic focal necrotic lesions in the liver of mice on intraperitoneal (i.p.) inoculation, whereas type 1 strains only occasionally produce a few tiny lesions hardly visible to the naked eye. It was suggested that this observation might be used as a simple and reliable biological method for the differentiation between HSV type 1 and type 2. The present study was undertaken to investigate some biological conditions that may influence the outcome of the test.

MATERIALS AND METHODS

Viruses. Strain MacIntyre (type 1) and strain MS (type 2) were used as type strains in all experiments. In addition, a total of 7 type 1 strains and 11 type 2 strains were used in various experiments. Their origin, site of isolation, serological typing, passage history and ability to produce focal necrotic hepatitis in mice have been described previously (Magnusson *et al.* 1974).

Stocks of cell-free virus were produced in human embryonic lung-cell cultures (HEL) grown in Eagle's minimal essential medium (MEM) supplemented with 2 per cent calf serum, antibiotics (200 IU penicillin, 200 μg streptomycin, and 2.5 μg mycostatin per ml) and 0.08 per cent sodium bicarbonate. The cultures were harvested when 90 per cent of the cells showed cytopathic effect by two cycles of freezing and thawing followed by gentle centrifugation (650 G/ 10 min). The supernatant was stored in 1 ml plastic ampoules at 70° C until use. Virus dilutions for animal inoculation and virus titration were made in MEM containing 5 per cent calf serum, bicarbonate and antibiotics as indicated above. Viruses were titrated by a plaque method by adding 0.1 ml of serial 10-fold dilutions of stock virus to duplicate plastic Petri dish cultures of HEL cells. After 1 hour of adsorption at 37° C, the monolayer was covered with MEM containing 5 per cent calf serum, antibiotics, bicarbonate, and 1 per cent methyl cellulose. The cultures were placed in a humidified 5 per cent CO_2 atmosphere at 37 C, and after 2 days, the plaques were counted following staining with methylene blue. The type 1 virus titres ranged from 3×10^7 to 8×10^8 p.f.u. per ml, while the type 2 virus titres were generally lower ranging from 2×10^6 to 3×10^7 p.f.u. per ml.

Experimental animals. Thirteen different strains of mice were used. For most experiments either conventional bred Ssc AH mice from the State Serum Institute, Copenhagen, or NMRI/BOM SPF mice from G1 Bomholtgaard Laboratory Animal Breeding and Research Centre, 8680 Ry, Denmark, were used. The following inbred strains: Balb/c/A/BOM, CBA/J/CR/BOM, C3H/Tif/BOM, C57/BL/6J/BOM, C57/BL/10ScCr/BOM, DBA/2/J/BOM, ST/aFi/BOM, NZB/Cr/BOM and GR/Fi/BOM, all SPF animals, were also purchased from this centre.

Furthermore the series of mutant stock used included hr/hr/BOM and nu/nu Balb/A/BOM, the latter a nude mouse with aplasia of the thymus in the 6th backcross generation with Balb/c/A/BOM. All experiments were performed with mice aged 3-4 weeks unless otherwise stated in the text.

In addition, the following conventional bred experimental animals were also used: albino rabbits (Ssc-CPH), golden hamsters (Ssc GH) and guinea pigs (Ssc AL), all purchased from the State Serum Institute, Copenhagen, as pregnant animals, the offspring of which were of different ages when used. Finally young rats from a local stock of F1 s produced from inbred Hooded and AS rat strain were used.

Experimental procedure. The animals received intraperitoneal inoculation into the left lower quadrant with an undiluted virus suspension unless otherwise stated. Mice, rats and hamsters were given an inoculum of 0.1 ml while rabbits and guinea pigs received 0.5 ml per animal. After 4 days, the animals were killed and the liver examined carefully for macroscopic lesions. The size of the focal necrotic lesions was scored either as large or tiny, i.e. 0.5–1 mm and less than 0.2 mm in diameter respectively.

RESULTS

Influence of the Age of the Mice

Groups of mice of different ages were inoculated i.p. with 2 × 10 p.f.u. of HSV type 1 or type 2. It appears from Table 1 that in the conventional bred AH mouse strain, a clear-cut upper age limit for the ability of the type 2 strain to produce large focal necrotic liver lesions (Fig. 1 a) was not found, although the number of mice with lesions as well as the number of lesions in individual mice declined in the upper age groups. Using NMRI/BOM SPF mice, an upper age limit for the development of macroscopic lesions in the liver was found to be 4 weeks. Moreover in these mice, the lesions were generally less numerous than in AH

TABLE 1. *Number of Mice of Different Ages Showing Liver Lesions after Intraperitoneal Inoculation (2 × 10⁴ p.f.u. of HSV Type 1 (MacIntyre) and Type 2 (MS)*

Age (weeks)	Conventional AH Type 1	Conventional AH Type	NMRI SPF Type 1	NMRI SPF Type 2
1	0/9	10/10	ND	ND
	2/10*	9/9	ND	ND
3	3/10*	10/10	0/15	10/15
4	1/10*	7/10	0/15	6/15
5	1/9*	10/10	0/5	0/5
6	0/10	5/10	0/5	0/5

* Tiny lesions.
ND not done.

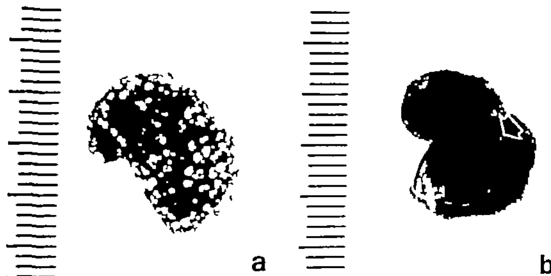


Fig 1 Livers from mice inoculated intraperitoneally with herpesvirus and sacrificed 4 days later (a) HSV type 2 numerous focal necrotic lesions *are seen on the surface of the liver (b) HSV type 1:* a few tiny lesions are seen on the liver margin (arrow) Magnification × 2.5

mice of comparable age The type 1 strain produced only a few tiny lesions in a few AH mice. These type 1 lesions were almost exclusively located *as a rim on the anterior* margins of the liver (Fig 1 b)

Influence of the Strain of Mice Used

As the previous experiment had shown an obvious difference in the susceptibility of two strains of mice to i.p. inoculation with HSV type 2, an experiment was undertaken to study the influence of the genetic conditions of mice on the outcome of the test. Thirteen different strains of mice aged 3–4 weeks were inoculated i.p with 3×10^4 p.f.u. of HSV type 1 or type 2. Four days later a spectrum of reactivities to the two virus types was seen (Table 2) In most strains of mice, HSV type 2 induced many large hepatic foci of necrosis. Three mouse strains were however totally negative. The few tiny lesions seen in some mice inoculated with the type 1 strain were easily distinguished from the type 2 lesions.

The inability of HSV type 2 to produce large focal necrotic liver lesions in the GR mouse strain was further investigated, using a wider range of type 2 strains. Twelve different HSV type 2 isolates previously shown to give focal necrotic lesions in AH and NMRI strains of mice were inoculated i.p. *in groups of 5 GR mice aged 3–4 weeks.* Only 2 virus strains produced a few large lesions, while the others were either entirely negative (2 strains) or showed a few tiny type 1 like lesions (8 strains)

TABLE 2 *Number of Mice Showing Liver Lesions after Intraperitoneal Inoculation of 3 × 10^4 p.f.u. of HSV Type 1 (MacIntyre) and Type 2 (MS)*

Mouse strain	Type 1	Type
AH	3/5	3/5 (15)‡
NMRI	1/5	4/5 (10)
Balb/c	2/5	3/5 (7)
CBA	2/5	5/5 (30)
C3H	3/5	5/5 ([illegible]0)
DBA	0/5	5/5 (20)
ST	0/5	5/5 (50)
hr/hr	0/5	5/5 (40)
NZB	0/5	2/5 ( )
C57/Bl/6	0/5	1/5 (1)
C57/BL/10	0/5	0/5
GR	0/5	0/5
nu/n Balb/c	0/5	0/5

Tiny lesions.
‡ Average number of lesions.

TABLE 3. *Number of Suckling Rats and Hamsters 7 and 14 Days Old Respectively Showing Liver Lesions 4 Days after Intraperitoneal Inoculation of Different HSV Type 1 and Type 2 Strains*

Virus strains	Inoculum (p.f.u. × 10[illegible])	Rats	Hamsters
Type 1			
MacIntyre	30	0/5	4/5
121	100	0/5	ND
129	100	0/5	2/5
179	50	0/5	ND
150	100	0/5	1/5
326	40	0/4	ND
OJ-H	70	0/5	ND
OJ-R	600	0/4	ND
Type 2			
MS	30	4/4	5/4
415	35	3/4	5/4
Men	2	3/4	3/5
A4069	1	3/4	2/4
A920	15	4/4	ND
A862	6	3/4	ND

Tiny lesions.
ND not done.

Influence of the Virus Inoculum

In order to study the minimum number of infective virus units needed to produce focal necrotic hepatitis in mice, NMRI/BOM SPF mice aged 3–4 weeks were inoculated i.p. with 0.1 ml of serial 10-fold dilutions of 2 HSV type 1 strains and 6 HSV type 2 strains. With 5 of the 6 type 2 strains, doses as small as 10^2 p.f.u. were sufficient to produce large focal necrotic lesions in the liver of half of the animals inoculated while in the case of the last strain the minimum dose was 10^3 p.f.u. Using 10 p.f.u. no lesions at all were recorded with any strain. One of the type 1 strains (MacIntyre) produced a few type 1 lesions when 10 to 10^4 p.f.u. were inoculated, whereas the other one was totally negative.

Use of other Laboratory Animals

In preliminary studies of rabbits, rats, hamsters, and guinea pigs of different ages it was found that in 1-day-old rabbits and in rats and hamsters of ages up to 7 and 14 days, respectively liver lesions similar to those seen in mice were regularly found on i.p. inoculation of HSV type 2, whereas even young guinea pigs were almost completely refractory.

As 1-day-old rabbits, although positive, were found to be very inconvenient to handle, suckling rats and hamsters aged 7 and 14 days, respectively were further studied. Table 3 shows the results of the inoculation of different HSV type 1 and type 2 strains in these animals. Both species of animals were found suitable for the differentiation between the inoculated HSV types on the basis of the development of hepatic lesions.

DISCUSSION

The purpose of the present study was to examine the influence of some biological conditions on the outcome of the focal necrotic hepatitis test for the differentiation between herpes simplex virus types 1 and 2.

It was found that in most, although not all, of 13 different strains of mice large focal necrotic liver lesions regularly developed on intraperitoneal inoculation of HSV type 2, whereas none showed such reaction to HSV type 1. Twelve out of the 13 strains of mice were reared under identical SPF conditions and were of the same age when used. Furthermore, as susceptibility to HSV type 2 liver infection was found to be different in the 9 inbred strains used, it is likely that this difference is genetically determined and may either be a property of individual cells or depend on the immune responsiveness of the animal (*Allison* 1965). Recently *Lopez* (1975) provided evidence suggesting that resistance to fatal HSV type 1 infection in inbred mouse strains is dominant, at least 3 genes being involved. This resistance was not found to be a property of structural cells, but is probably immunologically mediated.

The age-dependent resistance to HSV type 2 liver disease observed in the NMRI/BOM SPF mouse strain seems to be equivalent to the increased resistance to extraneural inoculation of HSV type 1 which develops in mice with age (*Johnson* 1964). Johnson

found that the resistance was caused by differences in the ability of the peritoneal macrophages from young and old animals to replicate the virus. The basic difference in liver involvement between HSV types 1 and 2 seems, however, not to be an age-related property since even 1-week-old mice do not show large liver lesions if the type 1 virus strain is used. The less marked upper age limit for liver involvement seen in conventional bred AH mice may be genetically determined, but differences in breeding conditions should also be taken into account.

The nu/nu Balb/c, a nude mouse with aplasia of the thymus, was among the mouse strains which did not show focal necrotic hepatitis on inoculation with HSV type 2. This mouse strain has experienced 6 back cross matings with Balb/c, and thus differs from the latter strain only in respect of $(1/2)^6$ i.e. 1.6 per cent of the genetic constitution. As it was found that the Balb/c mouse did react with the development of focal necrotic lesions, this observation might point to the focal necrosis as an immunological response to HSV type 2 mediated by T lymphocytes. Studies of nu/nu mice possessing a higher degree of genetic homology with Balb/c mice are, however, needed before it can be presumed that absence of lesions is related to the nu gene and perhaps also to thymic aplasia.

The studies of the minimum infective dose of virus required for the test showed that doses as small as 10^2 to 10^3 p.f.u. of HSV type 2 in 0.1 ml of diluent are sufficient. These infectivity titres are easily achieved in cell cultures used for HSV isolation and hence, virus titration has not to be carried out in advance. The costs and time required to determine the type may thus be appreciably reduced.

Other laboratory animals such as suckling rats and hamsters were found suitable. In these animals, an age-dependent resistance to HSV type 2 liver infection was also seen. The animals are easy to handle and will survive for sufficiently long periods of time for the liver lesions to develop. The results were found to be most easily interpreted in rats, as these showed no visible lesions at all if the type strains were used, while tiny type 1 lesions were very often seen in hamsters.

In a recent paper from another laboratory (*Vahlne et al.* 1975) the focal necrotic hepatitis test was compared with other established typing procedures. The method was found to give reproducible results, and there seemed to be a good correlation with results obtained by serological typing. As the present study of the biological conditions influencing the test has shown only a few limitations for its practical application, it should be considered a useful tool in the typing of HSV.

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BRIEF REPORTS

REACTIVITY OF RABBIT IgG-FRAGMENTS IN SINGLE-RADIAL IMMUNODIFFUSION

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Haaheim, L. R. Reactivity of rabbit IgG-fragments in single-radial-immunodiffusion. Acta path. microbiol. scand. Sect. B, 84 159–161 1976.

The previously described method of assaying the antibody response to influenza virus by single-radial-immunodiffusion is based on the formation of opalescent zones when immune sera are allowed to diffuse into gels containing large amount of purified influenza virus. The zones are not thought to be the result of immune aggregates of virus particles and antibody since the virus is immobile in the gel and the average distance between two neighbouring particles is approximately 50 times the virus diameter. The present study investigates how different rabbit IgG-fragments react in this type of test. It was found that IgG F(ab') and reduced (monovalent) IgG gave rise to distinct zones of opalescence. Fab' on the other hand, produced weak and hazy zones which could not be quantitated with any accuracy. We conclude that the most likely mechanism responsible for the zones is a light-scattering effect caused by antibodies attached to the viral surface and that the quality of the opalescence to some extent seems to be dependent on the Fc-fragment.

Key words: IgG-fragments; single-radial-immunodiffusion; rabbit.

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Schild et al. (5) recently described a method for measuring the antibody response to influenza virus. Serum samples were added to wells cut in an agarose gel containing a large amount of intact purified influenza virus. After the sera had diffused into the gel zones of opalescence were observed. The area of the zone formed was shown to be proportional to the amount of antibodies to the viral surface antigens. The mechanism responsible for the appearance of zones is not known. Since the viral particles cannot diffuse in the gel and the average distance between two neighbouring particles in the gel was found to be about 50 times the particle diameter (4), the zones are not thought to be the result of aggregates of virus particles and specific antibodies. In order to investigate what causes these zones, IgG from rabbits immunized with influenza virus was subjected to pepsin and/or reducing agents and tested for their ability to form zones of opalescence in single-radial-immunodiffusion.

Materials and Methods

Virus. The recombinant strain MRC8 (H3N2) kindly provided by Dr. G. C. Schild, World Influenza Centre, London, was grown in embryonated hens eggs and purified as described by *Schild & Pereira* (4).

Sera. Rabbits were given two subcutaneous injections of 500 μg of purified virus mixed with Freund's complete adjuvant at one week interval. The rabbits were bled 4 weeks after the last injection. The serum used in the following experiments contained $\log_2$ 5.0 anti-haemagglutinin units/ml. Haemagglutination-inhibition tests were performed according to standard procedures (7).

Purification of IgG. The immune serum was mixed with the anionic exchanger Sephadex A 50 (Pharmacia, Sweden) which had been preswollen

and equilibrated with 0.1 M Tris-HCl buffer pH 8.0. One g of wet gel was used per ml of serum. After stirring carefully for 1 hour at 4 C, the mixture was filtered (Whatman filter paper No 1) and the gel was washed with 5 volumes of cold buffer. The IgG was precipitated from this filtrate by adding dropwise an equal olume of cold, saturated $(NH_4)_2SO_4$. After 2 hours at 4 C the mixture was centrifuged and the pellet resuspended in distilled water. The solution was dialyzed against running tap water for 2 hours and thereafter dialyzed against 200 volumes of 0.015 M phosphate-buffered saline (PBS) pH 7.3 overnight at 4 C.

Pepsin digestion of IgG. The IgG preparation was dialyzed against 0.1 M acetate buffer pH 3.5 overnight in the cold. Pepsin (Nutritional Biochemicals Corp U.S.A. twice crystallized) was then added at an enzyme to protein ratio of 1:30 (w/w). After incubation for 16 hours at 37 C with stirring, saturated tris(hydroxymethyl-)aminomethane (Sigma, U.S.A.) was added dropwise until the preparation reached a pH 7.5. Undigested IgG was removed by absorption with staphylococci (2). The supernatant, consisting of $F(ab')_2$, was dialyzed against PBS overnight at 4 C.

Reduction of IgG and $F(ab')_2$. The IgG- and $F(ab')_2$-preparations were first deoxygenized by slowly bubbling N_2 through the solutions. Then 2 mercaptoethanol (Sigma) was added to a final concentration of 0.05 M. After 2 hours at room temperature, iodoacetamide (Sigma) was dded in 20 per cent molar excess over reducing agent and left for 1 hour at room temperature. The preparations were then dialyzed against PBS overnight in the cold.

In order to remove aggregates and grossly de- ured material, the four different preparations, C $F(ab')_2$, Fab' and reduced and alkylated IgG ere centrifuged at 30,000 rev/min for 1 hour in pinco L50 preparative ultracentrifuge using a SW50.1 rotor. The upper third of the supernatants was removed and used in the further experiments.

Assessment of purity. The different IgG-derived fractions were run in 7 per cent polyacrylamide gels with sodium dodecyl sulphate according to *Skehel & Schild* (6). As markers, bovine serum albumin (MW 64,000) and rabbit IgG (MW 150,000) were run in parallel gels. The purity of each preparation was determined by scanning the stained gels in a Chromoscan spectrophotometer (Joyce-Loebl, England) and found to be better than 90 per cent.

Protein d terminat on. The Micro-Kjeldahl method was used (1).

Immun o-double-diff sion (IDD) and single radial-immunodiffusion (SRD). IDD and SRD were carried out according to *Schild* (3) and *Schild et al.* (5) respectively. In order to reduce non-covalent interaction between the monovalent IgG fragments, IDD and SRD were performed in the presence of the detergent Sarkosyl NL35 (kindly donated by C ba-Geigy England) at a concentration of 0.05 per cent (v/). This amount of detergent did not seem to interfere with the antigen-antibody reactions nor did it appear to solubilize the virus particles in SRD. The former statement is based on the finding of precipitin lines in IDD and zones of opalescence in SRD with detergent treated IgG molecules, whereas the latter statement is supported by the lack of opalescence in SRD when antiserum to the inner structures of the irus was employed. The detergent also hindered non-covalent interaction between the monovalent IgG fragments since the haemagglutination-inhibition titers observed for the different IgG preparations were found t be the same before and after the detergent treatment with the exception of monovalent IgG showing an 8-fold decrease in titer.

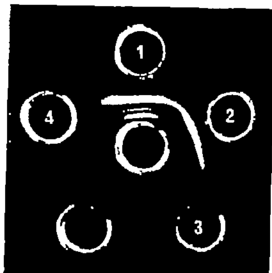


Fig. 1. Immuno-double-diffusion with IgG-derived fractions and MRC8 v rus.

Centre well: MRC8 irus, approx. 10 mg/ml of virus protein, treated with the detergent Sarkosyl NL35 at 1 per cent (/) final concentration. 1 Intact IgG stock solution 9.0 mg/ml, diluted 1:2. 2 $F(ab')_2$, 4.5 mg/ml. 3 Monovalent IgG 4.6 mg/ml. 4 Fab' 3.3 mg/ml. All antibody preparations were treated with the detergent Sarkosyl NL35 at 0.05 per cent (v/v) final concentration.

Results and Discussion

Fig. 1 shows that intact IgG and $F(ab')_2$, in contra t to monovalent IgG and Fab' are able to precipitate the ral antigens in IDD. This is due to the divalent nature of IgG and $F(ab')_2$.

In SRD IgG $F(ab')_2$ and even monovalent IgG all produced zones of opalescence (Fig. 2). How-

ever Fab gave rise to a very weak and hazy zone which was impossible to measure with any accuracy

Since a definite zone of opalescence was observed with the monovalent IgG this leads to the conclusion that the zones in SRD cannot be caused by immune aggregates of viral particles and antibody in the gel. Furthermore, since there is such a great difference in the intensity of zones caused by IgG and $F(ab)_2$ the quality of the opalescence must in some way depend on the Fc-fragment. The zones in SRD may therefore be due to a light scattering effect caused by the antibodies attached to the viral surface. This phenomenon seems to be dependent on the tertiary structure of the different types of antibody fragments involved.

This test system has been found to be accurate, reproducible and sensitive and able to distinguish between immune sera differing only by approx. 10 per cent in antibody content (5) It is thus a good method of estimating small differences in antibody response. In experiments involving the investigation of biological activity of different sera and even different fragments of immunoglobulin subclasses, the SRD-test stands out as a valuable tool. But one has to bear in mind that the zone size for monovalent IgG is somewhat reduced compared with the zones caused by IgG and $F(ab)_2$. It therefore appears that IgG and F(ab) can be quantitated (as mm² of opalescent zone) and compared in the same immunoplate, whereas a direct comparison with monovalent IgG is hardly advisable unless an internal standard of monovalent IgG is employed.



Fig 2 Single-radial-immunodiffusion with MRC8 virus and IgG-derived fractions. All preparations were used undiluted, see legend to Fig 1 The actual diameter of the wells is 4 mm.

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ISOLATION OF HYALURONIC ACID FROM CULTURES OF STREPTOCOCCI IN A CHEMICALLY DEFINED MEDIUM

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Kjems, E. & Lebech, K. Isolation of hyaluronic acid from cultures of streptococci in a chemically defined medium. Acta path. microbiol. scand. Sect. B, 84 162–164 1976.

A haemolytic streptococcus (Lancefield's group A) has been cultivated in a chemically defined medium. About 300 mg hyaluronic acid of a high degree of purity was isolated per litre of this culture.

Key words Streptococci hyaluronic acid isolation chemically defined medium.

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In this paper a method is described for preparing purified hyaluronic acid from cultures of a haemolytic streptococcus, Lancefield's group A, type 12 strain K56 (4)

The strain was grown in a chemically defined medium developed in the Media Department, ...ns Seruminstitut This medium is a simplified ...on of that developed previously (*Lebech K.*, unpublished results, 1967) Table 1 shows the composition of the chemically defined medium used. A dry stock mixture and stock solutions were prepared according to *Williams* (8)

The medium was sterilized by millipore filtration or by autoclaving at 110°–112 C for 35 minutes. When autoclaving, K_2HPO_4, $NaHCO_3$ and $CaCl_2$ were not added until just before the medium was inoculated with strain K56.

Three flasks, each containing about 3.4 litres of the medium, were inoculated with 3.4 ml of an overnight culture of K56 grown in the chemically defined medium. After incubation for about 48 hours at 36 C, the culture was placed in steam at 100° C for about 30 minutes. The killed culture in the three flasks was filtered through two filters (Carl Schleicher & Schüll paper no. 597 Ø 24 cm) each into two flasks. The filtrate from all six flasks was transferred to three steel buckets, so that each contained about 3 litres. About 6 litres of ethanol (99.9 per cent) were poured into each bucket. The contents were stirred and the buckets placed at room temperature until the following day

The resulting sediment consisted of crude hyaluronic acid. Most of the supernatant was discarded and the crude hyaluronic acid suspended in the remaining supernatant, transferred to three stainless steel beakers and centrifuged for 30 minutes at 2100 *g* The supernatant was discarded, and the sediment in each beaker was suspended 140 ml of warm (80–100 C) sodium acetate buffer (0.1 M, pH 6, with 0 15 M NaCl added) After careful homogenization the suspension was allowed to stand at room temperature overnight. After centrifugation the viscous fluid was poured into a 2 litre glass beaker and ? olumes of 1 per cent cetylpyridinium chloride was added (5) The sediment was llowed to stand for 30 minutes after thorough mixing After further centrifug tion the supernatant was discarded nd the sediment was dissolved in 420 ml 0.5 M NaCl contai g 4 per cent ethanol (5) The suspension was arefully mixed and placed i a water-bath at 100 C until dissolved. Subsequently the solution w centrifugated for a f rther 30 minutes in order to ensure that t was perfectly clear The pure hyaluronic acid was precipitated with 2 olumes of ethanol (99 9 per cent) In order to remove the last traces of moisture the resulting large white flakes of

TABLE 1 *Composition of Chemically Defined Medium Used for Culturing Streptococcus Strain K56*

Amino acids + glucose	mg/ml	Purines-pyrimidine	mg/ml
L-alanine	0.2	Adenine	0.02
L-arginine HCl	0.24	Guanine	0.02
L-asparagine H_2O	0.22	Uracil	0.02
L-aspartic acid	0.2	B vitamins	µg/ml
L-cystine	0.1	DL-Ca-pantothenate	0.5
L-glutamine	0.35	Riboflavin	0.5
L-glutamic acid	1.0	Thiamine HCl	0.5
Glycine	0.4	Niacin	1.0
L-histidine	0.4	Pyridoxamine 2 HCl H_2O	1.0
L-hydroxyproline	0.05	Pyridoxal HCl	1.0
L-isoleucine	0.2	Folic acid	0.005
L-leucine	0.2	Biotin	0.0025
L-lysine HCl	0.25	p-aminobenzoic acid	0.1
L-methionine	0.2	Salts	mg/ml
L-phenylalanine	0.2	K_2HPO	0.5
L-proline	0.2	KH_2PO	14.1
L-serine	0.2	MgSO 7 H_2O	0.2
L-threonine	0.2	$FeSO_4$ 7 H_2O	0.01
L-tryptophan	0.2	MnSO 4 H_2O	0.01
L-tyrosine	0.2	NaCl	0.01
L-valine	0.2	NaC_2H_3O 3 H_2O (sodium acetate)	10.0
Glucose	10.2	NaHCO	0.5
		$CaCl_2$ 2 H_2O	0.039

TABLE 2. *Analysis of Hyaluronic Acid Preparations from Bacteria and from Umbilical Cords. Per Cent Dry Weight*

Preparation	Glucuronic acid	Glucosamine	Acetyl	Nitrogen	Ash	Protein
Mar 7 1973 from bacteria	42.3	36.1	8.5	2.85	9.2	<0.2
Feb. 20, 1974 from bacteria	41.2	35.4	8.0	2.87	11.1	<0.3
Oct. 22, 1969 from umbilical cord	38.9	35.2	8.3	3.0	13.8	2.0

Glucuronic acid was determined by *Bitter & Muir* (1) carbazol methods with d-glucuronic acid (Fluka AG, Switzerland) as standard.
Glucosamine. Samples were hydrolyzed with 8 N HCl at 95 C for three hours (7). The hydrolysate was examined using *column chromatography according to the method of Spackman et al* (6) using Beckman special particle resin type M 81.
Nitrogen was determined by the Kjeldahl method of *Brecher* (2).
Ash. Samples were incinerated   Heraeus electric oven at 460° C until the weight was constant.
Protein was determined by the biuret method (9).
Calculated values, assuming that all the glucosamine is N-acetylated.

hyaluronic acid were transferred to a glass beaker containing ethanol (99.9 per cent) in which they were allowed to stand until the following day. The white flakes were then placed in ether (to remove the alcohol) and after at least 2 hours they were transferred to a glass petri dish which was left open until the following day. The white flakes were weighed and stored at room temperature. We isolated approximately 3 g hyaluronic acid from about 10 litres of culture.

Table 2 shows the results of chemical analysis of two preparations of hyaluronic acid chosen at random from batches which were prepared with one year interval. An analysis of umbilical cord hyaluronic acid prepared as described by *Faber* (3) is also shown.

Since 1973 we have used hyaluronic acid of bacterial origin instead of hyaluronic acid from umbilical cord in the routine determination of anti-streptococcal hyaluronidase in human serum samples.

The use of a chemically defined medium for the preparation of hyaluronic acid ensures reproducibility in the yield and degree of purity of the hyaluronic acid. Complications associated with the fact that an undefined substrate contains substances of high molecular weight, e.g. protein, are avoided. Protein present in the chemically defined medium which contains hyaluronic acid *after* growth of the streptococcus strain is solely of bacterial origin.

The chemically defined medium seems to offer many possibilities among which is its usefulness for examining the antigens of streptococci since it is an antigen-free medium.

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THE VACCINIA VIRUS HAEMAGGLUTININ ENZYME SENSITIVITY AND SOME PHYSICOCHEMICAL PROPERTIES

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Gurvin, I. & Haukenes, G. The vaccinia virus haemagglutinin. Enzyme sensitivity and some physicochemical properties. Acta path. microbiol. scand. Sect. B, 84: 165–169, 1976.

Purified vaccinia virus haemagglutinin (VHA) was found to be sensitive to trypsin both with regard to haemagglutinating capacity and antigenic property. Phospholipase C and lipase had no effect on the haemagglutination (HA). Both the HA and the antigenic property thus depend on the integrity of the protein part. The loss of HA after treatment with trypsin may be due to breakdown of an essential protein or to secondary rearrangement of the whole structure, including the lipid part. Cells infected with an HA negative mutant of vaccinia virus also lacked the antigenic part of VHA. The sedimentation constant of VHA from HeLa cells was about 50.

Key words: Vaccinia; haemagglutinin; enzyme treatment.

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The vaccinia haemagglutinin (VHA) has been found to be sensitive to phospholipase C (7) leading to the conclusion that a phospholipid is essential for the haemagglutinating property. Trypsin treatment of a partly purified VHA from chick chorioallantoic membranes had no effect on the haemagglutination (HA) (9). In another study, however, treatment of vaccinia-infected Hep-2 cells with trypsin led to loss of the haemadsorbing capacity of the cells, and almost no haemagglutinin could be extracted (1). The latter authors conclude that the VHA arises from the cytoplasmic membrane.

VHA obtained from cells of different species has been found to differ in density range and molecular size (5), suggesting that the lipid part is of host origin.

We have examined the enzyme sensitivity of a VHA purified from HeLa cells, and our results differ in several respects from those reported above. The VHA material has also been studied physicochemically with regard to density range and molecular size.

MATERIALS AND METHODS

Virus strains

Three vaccinia virus strains have been used: The smallpox vaccine strain, Statens Seruminstitut, Copenhagen; the IHD-J strain which is haemagglutinin positive and cell fusion negative; the IHD-W strain which is haemagglutinin negative and cell fusion positive (3). The 2 mutant strains were obtained from Dr. *Yasuo Ichihashi*, Kyoto, Japan. Unless otherwise stated the Copenhagen strain was used.

Tissue Culture

The VHA was produced in HeLa cells (Bristol strain, calf serum adapted, Flow Laboratories, Irvine, Scotland) Preparation of antigen for immunization was made from a harvest of vaccinia infected rabbit lung cells. Details of the cell culture procedures have been given earlier (2)

VHA

The VHA was obtained by a 2-step centrifugation procedure and kept in 0.1 M glycine buffer pH 8.4 The purification procedures and some properties of the VHA have been reported in (2)

Antisera

Rabbits were immunized intravenously with vaccinia virus after a second passage in rabbit lung cells, the serum added to the culture medium being obtained from the rabbit to be immunized (cf (2))

HA and HA Inhibition (HI)

The titrations were performed by the standard procedures as described in (2) except in the trypsin experiments. Small amounts of trypsin (about 12 μg/ml) caused the vaccinia-agglutinable erythrocytes to agglutinat when incubated at 37 C. This agglutination was negligible at 4 C, especially when 1 per cent calf serum was added to the buffer Under these conditions the vaccinia-induced HA was unaffected or slightly lowered, maximally one titre step on two-fold serial dilution.

Enzym and Enzyme Treatment

The trypsin used was Crystalline Trypsin Novo (dialysed and lyophilized) from Novo Industri A/S Copenhagen. The phospholipase C (*ex Clostridi m perfringens*) and lipase (*ex* wheat germ) were both from Koch-Light Labs. Ltd., Colnbrook, Bucks., England) All enzyme treatments were carried out for 1 h at 37 C and at a final concentration of 0.1 per cent. An 0.05 M tris buffer pH 8.0 was used for the trypsin treatment, and 0 1 M glycine buffer pH 8.4 for phospholipase C and lipase treatment.

Periodate Treatment

One volume of the VHA preparation (dialysed overnight against PBS to remove sucrose) or vaccinia-infected cells in saline buffered to pH 7 2 by 0 06 M phosphate (PBS) was mixed with two volumes of an 0.0167 M solution of KIO and left for 15 min at room temperature. The reaction was terminated by addition of one volume of 3 per cent glycerol in PBS

Ultracentrif gation

All centrifugations were performed in a Spinco ultracentrif ge, Model L65B. The density of the VHA was determined by adding sucrose to VHA to a final concentration of 50 per cent (W/V) and placing the mixture in a centrifugation tube over a 40 to 10 per cent sucrose gradient. The sedimentation coefficient (S) was determined from the clearing factor (k) as specified in the Spinco manual for different densities of the solvent and for individual rotors. $S = \frac{k}{t}$ t being the time in hours needed to get the VHA into the pellet.

EXPERIMENTS AND RESULTS

Trypsin

Our results indicate that trypsin inactivated the HA activity As seen from Table 1 only 6 per cent of the HA activity was left after trypsin treatment for 60 min at 37 C

TABLE 1 *Trypsin Treatment of VHA*

	HA titre 0 min	HA titre after 60 min at 37 C
VHA + buffer	800	800
VHA + trypsin	800	50
Buffer + trypsin	<50	<50

As reported earlier the HA-active material in VHA covered a wide range of densities, 1 08–1 18 g/cm VHA samples representing densities from 1 08–1 18 g/cm were treated with trypsin. The HA titre of each sample was found to be reduced to the same extent, indicating the same degree of sensit vity to trypsin.

TABLE 2 *Absorption of HI Antibod es*

Antiserum absorbed with	HI titre after absorption with untreated cells	HI titre after absorption with trypsinized cells
HeLa	640	640
HeLa inf with IHD-W (HA neg.)	640	640
HeLa inf with IHD-J (HA pos.)	40	640

The trypsin sensitivity of the antigenic part of VHA was examined by absorption of anti-vaccinia serum with vaccinia infected HeLa cells. Table 2 shows that trypsin treatment of HeLa cells infected with the HA positive strain (IHD-J) of vaccinia virus destroyed the ability of the cells to absorb the anti-HA antibodies. Uninfected HeLa cells and HeLa cells infected with the HA negative strain did not absorb the anti-HA antibodies.

Phospholipase C

Treatment of VHA with phospholipase C gave no significant decrease in the HA activity. This result was somewhat surprising in view of earlier findings (7) of high sensitivity of VHA to this enzyme. The efficiency of the enzyme was controlled by an egg yolk test and by its haemolytic activity. Furthermore we examined whether any change in the density of VHA had occurred. The density of the enzyme-treated VHA was compared with that of untreated VHA by equilibrium floating centrifugation. It is seen from Fig. 1 that treatment with phospholipase C led to a decrease in the density values of the VHA. Untreated VHA exhibited a peak value at d = 1.12, in contrast to d = 1.07 for the phospholipase C treated material.

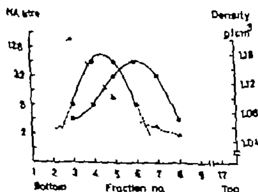


Fig. 1. Density distribution of VHA before and after treatment with phospholipase C. VHA was flotation centrifuged to equilibrium from 45 per cent sucrose into a 40 to 5 per cent sucrose gradient.
—●— untreated VHA
■ phospholipase C-treated VHA
▲ density of sucrose

Lipase

Treatment of purified VHA with lipase had no detectable effect on the HA activity or the density distribution of the VHA material.

Periodate Treatment

The haemagglutinating activity of the VHA preparation was not changed by treatment with periodate. The stability of the antigen of VHA was tested by periodate treatment of vaccinia infected cells. This treatment gave no reduction in the ability of the cells to absorb the HI antibodies of an antivaccinia serum.

Temperature and pH Stability

VHA in glycine buffer was exposed to 20°, 37° and 56° C for 1, 2 and 3 days. The results are shown in Table 3. It is seen that the VHA is relatively stable at 20° and 37° and up to 24 h at 56° C. The HA activity of VHA was examined at different pH values. It remained constant for 1 h at 37° C from pH 5 to pH 9 (Table 4). At pH values lower than 5 a rapid fall in the HA activity resulted, while a higher HA activity was repeatedly obtained at pH 10.

TABLE 3. *Heat Stability of VHA in Glycine Buffer pH 8.4*

Temperature	Hours 0	24	48	72
4° C	160	160	160	80
37° C	160	160	160	80
56° C	160	160	40	20

TABLE 4. *The Stability of VHA in Solutions with Different pH Values*

	pH 4.0	5.0	6.0	7.0	8.0	9.0	10.0
HA titre after 1 h at 37° C	10	160	160	160	160	160	160

11

Sedimentation Coefficient

The sedimentation coefficient of VHA was determined by the time of clearance as described under Methods and estimated to be 50 S (± 10 per cent)

DISCUSSION

Chemical and physicochemical data indicate that VHA is a lipoprotein and apparently a membrane-associated component (1) The lipid part has been considered to be essential for the HA capacity while the protein part possesses the antigenic property (4)

Our results with phospholipase C, which had no effect on the HA capacity are in contrast to earlier findings (7) Our enzyme preparation was shown to be highly effective on egg yolk and erythrocyte membranes. Furthermore, the lowering of the density range (Fig. 1) of VHA after treatment with phospholipase C shows that the enzyme was active on VHA, and the result is compatible with a splitting off of the more heavy hydrophilic residue at the C3 position on glycerol. We may thus conclude that the hydrophilic part of the phospholipid is not essential for the HA capacity

The lack of lipase effect on the HA is also in contrast to earlier findings (6) It is not whether the VHA possesses any neu- fat or whether neutral fat is accessible to e enzyme.

The effect of trypsin, which destroyed almost all the HA capacity is of considerable interest. The same effect has been demonstrated earlier (1) while others (9) found that this enzyme had no effect. Our findings clearly show that a protein or peptide is essential for the HA capacity of VHA.

The contrasting results reported above are difficult to explain. Host species differences in physicochemical properties of VHA are known to exist, and different procedures for preparation of the VHA may modify this material. Our results, however are in no way incompatible with the commonly accepted assumption that the VHA is a membrane-associated structure. When membrane proteins are destroyed reorganization of the membrane is known to take place, resulting in conformational changes. It is therefore still an open question whether the lipid or the protein part represents the binding site for the erythrocytes. Vaccinia-agglutinable erythrocytes are known to be highly agglutinable by lipids, e.g cardiolipin and some lipids from normal chick tissue (8) However these erythrocytes are obviously also sensitive to minor modifications of their membrane structure as seen from the agglutination caused by small amounts of trypsin (reported under Methods) It is unlikely that VHA causes agglutination as a result of a proteolytic effect on the erythrocyte membrane since the HA titre was independent of incubation temperatures between 4 and 37 C.

The trypsin sensitivity of the antigen part of VHA was clearly shown in the absorption experiments (Table 2) It is most interesting that the HA negative mutant strain, which did not induce HA, also lacked the HA associated antigen. This lends further support to the view that native VHA requires a protein backbone for its HA capacity

Our purified VHA from HeLa cells sedimented as a 50 S particle, a value which agrees well with the size of the particle found by electron microscopy (5)

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THE DIAGNOSTIC VALUE OF DETERMINATION OF IgM ANTIBODIES AGAINST *MYCOPLASMA PNEUMONIAE* BY THE INDIRECT IMMUNOFLUORESCENT ANTIBODY TEST

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Skaug, K., Eng, J., Ørstavik, I & Haug, K. W The diagnostic value of determination of IgM antibodies against *Mycoplasma pneumoniae* by the indirect immunofluorescent antibody test. Acta path. microbiol. scand. Sect. B, *84* 170–176, 1976

The indirect fluorescent antibody technique for specific IgM and IgG antibodies was applied to paired sera from 35 patients with current *M pneumoniae* infection, along with the complement fixation test. A roughly parallel increase in antibody titres in all the three tests was observed the ratio of IgM/IgG titres was, however higher in patients below 20 years of age than in older patients. Rises in antibody titres were regularly observed in spite of the fact that most of the patients were treated with tetracycline or erythromycin during the acute phase of the disease. Serum specimens from 15 other patients lacking clinical data of a recent *M pneumoniae* infection, but with stationary complement fixation titres, all showed IgG antibodies with stationary titres and, with the exception of three patients, a negative IgM fluorescent antibody test. Sera from 20 individuals lacking complement fixing antibodies were also without demonstrable IgG and IgM antibodies. Sampling of serum from 9 of the pneumonia patients was repeated. The last IgM positive sample was collected up to six months after onset of the disease and all were found to be negative in the IgM test 8 to 10 months after onset of the illness in these treated patients. The implications of these findings for the serological diagnosis of *M pneumoniae* infections are discussed.

Key words *Mycoplasma pneumoniae* IgM antibodies indirect immunofluorescence diagnostic value.

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The serological diagnosis of a current *M pneumoniae* infection is generally made by demonstrating a rise in titre of specific antibodies in paired serum samples drawn during the acute phase of the disease. The interval between the two serum samples ought to be at least one week, and in practical work a more rapid serological test yielding a tentative diagnosis is wanted.

The main purpose of the present work has been to examine the appearance the development and the persistence of *M pneumoniae* specific IgM serum antibodies, the

aim being to elucidate whether the presence of IgM antibodies in a single serum sample is of value in the diagnosis of a current *M pneumoniae* infection.

An indirect fluorescent antibody (FA) test is employed to compare the IgM antibody response with the IgG antibodies and the complement fixing (CF) antibodies.

MATERIAL AND METHODS

The material consisted of serum samples submitted to this laboratory for routine examination by CF tests with *M pneumoniae* antigen and various virus antigens. The sera had been kept frozen at −20 C for from two days up to one year before they were used in the present study. Sera from three different groups of patients were studied:

Group A consisted of 33 patients in whom a diagnosis of *M pneumoniae* infection was established on the basis of serological and clinical findings, and the date of onset of symptoms was known. At least two serum samples were available from all the patients. An additional follow-up series of blood samples were collected from 9 of the patients in order to determine the persistence of *M pneumoniae* specific IgM antibodies.

Sera from 27 of the patients showed a rise in CF antibody titre by 2 $\log_2$ dilution steps or more during the course of illness. Sera from the remaining 6 patients showed unchanged, mostly high CF antibody titres in the paired samples. These patients were included on combined clinical serological and family epidemiological grounds. In some of these patients the diagnosis was strengthened by a raised titre of cold agglutinins, in others by a family relationship with other patients under study. The commonly occurring intrafamilial spread of *M pneumoniae* (2, 5, 8) was used diagnostically in our study thus sixteen of the patients in group A belonged to 6 families. The clinical diagnosis was pneumonia in 25 patients, bronchitis in 4 patients and upper respiratory tract infection in 4 patients. Twenty-nine of the 33 patients were treated with erythromycin or tetracycline details of the treatment are given in Table 1. On the whole, group A is predominated by pneumonia patients who during the acute phase of the disease received adequate antibiotic treatment effective against *M pneumoniae*.

Group B consisted of an arbitrary selection of 15 patients who showed positive *M pneumoniae* CF tests with stationary titres of various heights during the routine examination clinical data compatible with a present *M pneumoniae* infection were lacking in all these patient

Group C was composed of 20 patients in whom both sera showed a negative *M pneumoniae* CF test.

Preparation of antigen for the indirect FA test The antigen employed in the FA test consisted of colonies of *M pneumoniae* "Bird" strain (6) transferred from agar blocks to glass slides by melting the agar in hot water as described by Clark et al. (4) The cultivation technique and the agar medium employed was as described elsewhere (6) Four to 5 days old agar cultures showing confluent growth of colonies were used. The *M pneumoniae* antigen was fixed on the glass slides in acetone for 10 minutes and then air dried. The preparations could be stored in containers at 4 C for several months without losing their specific reactivity in the FA test.

To keep the applicated serum drops in place on the prepared glass slides, small circular areas of the antigen, sufficient to be tested in the FA test, were surrounded by Tech-pen ink (Mark tex Corp)

Titration of serum samples The samples were titrated in twofold dilutions in the IgG and the IgM FA test, starting at 1:2. In the CF test, the samples were also diluted twofold, starting at 1.8 (1:4 in sera from children below 3 years of age)

The FA test procedure 0 1 ml of the serial serum dilutions were incubated on the prepared slides in a moist chamber at 37 C, one hour for determination of the IgG titre and 3 hours for determination of the IgM titre. Thereafter the slides were washed with four changes of phosphat buffered saline (PBS) for 15 minutes and then air dried. The test application areas on the slides were then covered with approximately 0.1 ml of the working dilutions of either the rabbit anti human IgG or the anti human IgM conjugates, and incubated and washed as previously described. The working dilutions of the conjugates, labelled with fluorescein isothiocyanate (D kopatts A/S) were 1 8 and 1:4 respectively The conjugates were diluted in Evans blue, diluted 1 10000 in PBS. Finally the slides were mounted in buffered glycerol at pH 9.0 A positive and a negative control serum were included in each test. The prepared slides were examined with incident light under an Ortholux microscope (Leitz) and only a typical, yellow-green fluorescence was taken into account. The serum antibody titre is expressed as the reciprocal of the highest dilution to give definite fluorescence.

To check the specificity of the conjugates, the 19S and the 7S fractions, after 10–40 per cent sucrose gradient centrifugation (SW 50, 1 32500 rev/min for 24 hours) of a *M pneumoniae* convalescent serum were examined in the FA test. No detectable cross reactions between the labelled anti IgG and the anti IgM were seen.

To determine the optimal serum IgG and IgM titre, positive serum was titrated and incubated on the slides for from ½ up to 4 hours. Every

TABLE 1 *Titres in the M. pneumoniae CF Test and IgG and IgM FA Tests in 33 Patients Suffering from a M. pneumoniae Infection. The Clinical Diagnosis and Data on Antibiotic Treatment are also Given*

Patient, Sex-age	Diagnosis	Days after onset of symptoms		Antibody titre		
		Antibiotic treatment	Serum sample	CF	IgG FA	IgM FA
F- 2	Bronchitis	E ?	?	8	4	2
			?	32	4	16
M-3	Pneumonia	Untreated	12	4	8	8
			25	128	128	128
M-5	Pneumonia	T* (5–13)	5	8	2	4
			17	128	32	32
M-6	Pneumonia	E (7–15)	8	<8	2	4
			22	512	256	512
M-6	Pneumonia	E (15–28)	15	16	8	4
			22	16	8	4
M-7	Pneumonia	E (5–10)	5	8	4	16
			23	≥64	128	32
M-7	Pneumonia	E (6–17)	6	8	4	8
			26	256	64	128
M-8	Pharyngotracheitis	E (2– 9)	2	<8	8	4
			27	128	64	32
F- 10	Pneumonia	E (9–17)	13	64	8	64
			17	128	64	128
F- 10	Stomatitis, Angina faucium	Untreated	2	<8	2	4
			11	16	16	32
F- 12	Pneumonia	E (9–19)	8	8	4	8
			26	256	64	256
12	Pneumonia	E (12–26)	10	32	16	64
			22	256	128	256
13	Pneumonia	E (2–10)	7	8	2	16
			22	64	4	32
M-14	Pneumonia	E (5–16)	5	<8	4	8
			16	≥64	64	256
F- 17	Pneumonia Mononucleosis	T (0– 7)	19	<8	16	8
			33	32	32	32
M-22	Pneumonia	T ?	33	128	64	32
			42	128	64	32
F- 23	Pneumonia	T (12–23)	12	<8	8	16
			25	≥64	128	512
M-26	Tracheobronchitis	T (5– ?)	6	<8	8	4
			19	16	64	32
F- 27	Pneumonia	E (7– ?)	6	<8	4	8
			16	16	32	32
F- 31	Pneumonia	T (3– 8)	7	8	32	8
			22	128	128	128
M-32	Gingivitis	Untreated	16	8	16	<
			28	32	64	16

TABLE 1 (continued)

Patient, Sex-age	Diagnosis	Days after onset of symptoms		Antibody titre		
		Antibiotic treatment	Serum sample	CF	IgG FA	IgM FA
F-34	Pneumonia	T (8–14)	8	<8	8	4
		E (14–18)	28	≥64	256	256
F-35	Tracheobronchitis	E (4–12)	4	8	2	<2
			34	16	16	<2
F-36	Pneumonia	T (3–9)	12	128	32	32
		T (12–16)	26	256	32	32
F-40	Pneumonia	T (8–15)	1	<8	2	4
			8	16	64	32
M-40	Pneumonia	T (5–15)	5	<8	2	<2
			25	64	32	<2
F-42	Bronchitis	T ?	?	8	4	4
			?	32	32	16
M 51	Pneumonia	Untreated	27	512	256	64
			37	512	256	64
M-56	Bronchitis	T (2–12)	2	<8	2	<2
			20	32	16	16
M-61	Pneumonia	E ?	?	<8	4	<2
			?	≥64	64	128
M-63	Pneumonia	T ?	?	<8	<2	<2
			?	16	4	8
F-65	Pneumonia	T+E ?	5	8	<2	<2
			11	256	16	4
F-71	Pneumonia	T (?–13)	14	<8	4	4
			20	16	64	16

E, erythromycin; T tetracycline.

half hour two slides were stained with the labelled anti IgG and the anti IgM, and the maximal titres were achieved after 1 and 3 hours respectively

The FA test, for determination of specific IgG and IgM antibodies against Herpes simplex (type 1) Varicella-zoster Parainfluenza (type 2) and Adenovirus was performed as previously described (10) The serum specimens were incubated in a moist chamber at 37° C, one hour for determination of the IgG titre and 3 hours for determination of the IgM titre. The virus antigens for the tests were grown in Rh 13, human embryonic fibroblasts, primary monkey kidney and HeLa cells, respectively

It has been shown that rheumatoid factor positive sera in some cases can give positive IgM fluorescence (9 11) All the *M pneumoniae* IgM positive sera were therefore tested and found negative in the Behringwerke A/G Latex test for rheumatoid factor

The CF test with *M pneumoniae* antigen, Bird" strain, was performed as previously described (7) but four 50 per cent units of complement were used in the present test.

RESULTS

The serological results and information about sex, age and antibiotic treatment of the patients in group A are presented in Table 1 In 31 of the 33 patients, IgM as well as IgG antibodies were demonstrated in both serum samples. The two patients, F 35 and M-40 were negative in the IgM FA test but showed a significant rise in titre in the IgG

TABLE 2 *Proportion between Specific IgM and IgG FA Titres in Relation to Age in Paired Serum Specimens from 33 Patients with a Recent* M. pneumoniae *Infection*

Age group, years	Number of patients with IgG and IgM FA titre*			Number of patients tested
	IgM>IgG	IgM=IgG	IgM<IgG	
2–20	6	9	0	15
21–71	1	10	7	18

* The titre differences are recorded as IgM>IgG or as IgM<IgG respectively when the IgM antibody titre is at least fourfold greater or less than the IgG antibody titre, either in the acute phase serum or in the convalescent serum or both.

FA test. On the whole the material showed a roughly parallel development of the IgM and IgG antibodies as well as of CF antibodies, during the acute phase of the *M pneumoniae* infection. The IgM/IgG ratio within group A, however was found to be correlated with age, as shown in Table 2. *In 6 out of 15 patients below 20 years of age*, the IgM FA titre showed at least a fourfold increase as compared with the IgG FA titre in one or both of the paired serum samples, whereas only 1 out of 18 patients older than 20 years exhibited the same antibody pattern. Conversely the IgG FA titre was found to exceed the IgM FA titre in 7 patients in the older age groups, but not in any of the patients below 20 years of age. During the first 7 days after onset of illness, the *M pneumoniae* specific IgM antibodies were demonstrated in 11 out of the 15 serum samples collected.

The paired samples from the 15 patients in group B lacking clinical data of a recent *M pneumoniae* infection all showed IgG

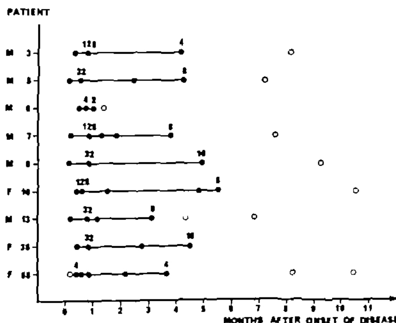


Fig 1 The persistence of the *M pneumoniae* specific IgM serum antibodies in 9 patients. The maximal titre and the IgM FA titre of the last positive sample are recorded at the corresponding serum specimens. ● represents a positive and ○ a negative serum sample.

antibodies with stationary titre and, with the exception of 3 patients, a negative IgM test. Two of the 3 patients showing a positive IgM FA test had a stationary titre and 1 patient presented a falling titre in the test under discussion. During the course of illness, one of these patients showed a significant rise in the CF and IgG FA test against Herpes simplex (type 1) and Varicella zoster but a negative IgM FA titre against the same antigens, while the other two patients showed a significant rise in the CF IgG and IgM FA tests against *Parainfluenza* (type 2) and *Adenovirus*, respectively

The 20 control patients in group C, with a negative CF antibody titre, all showed a negative *M pneumoniae* IgG and IgM FA test.

Fig. 1 illustrates the persistence of *M pneumoniae* IgM antibodies in 9 patients from group A. The last IgM positive sample was collected up to six months after onset of the disease and all were found to be negative in the IgM test 8 to 10 months after onset of the illness in these patients, most of whom had been treated. Patient M-6 had a low IgM FA titre, and it persisted for approximately 1 month only

DISCUSSION AND CONCLUSIONS

During the first 3–4 weeks of the disease, a roughly parallel increase in titres was found by the CF test and the IgM and IgG FA tests in the paired serum samples taken from the patients with *M pneumoniae* infections and known onset of symptoms (group A) Among 27 patients showing significant increase in the CF titres, 22 also showed an increase of two log₂ dilution steps or more both in the IgM and the IgG FA test. In addition, 3 patients showed an increase by one titre step in one or both of the FA tests. Among 6 patients showing stationary CF titres, 4 also exhibited stationary titres in both of the FA tests. Two patients, however showed negative IgM FA tests in both serum samples.

As group A is composed mainly of patients who received antibiotic treatment with tetracycline or erythromycin in the early phase of the disease, it can be concluded that such treatment does not prevent the rise in titres in the 3 tests under study The high degree of correlation between the FA tests and the CF test in this group of patients, as well as in the control group of patients yielding negative results in all the 3 tests (group C) supports the specificity of the IgM and the IgG FA tests in the serological diagnosis of *M pneumoniae* infections.

The observation that the IgM/IgG ratio was correlated with age is in accordance with the findings earlier reported by *Biberfeld* (3) who also offered the possible explanation that the patients in the older age groups might have experienced a re infection with *M pneumoniae*

The *M pneumoniae* IgM antibodies were demonstrated in 11 of the 15 samples collected during the first 7 days after onset of illness, thus suggesting an early appearance of the IgM antibodies.

As regards the 15 patients showing stationary *M pneumoniae* CF titres and lacking clinical evidence of a recent *M pneumoniae* infection (group B) all yielded a positive FA test for specific IgG antibodies, whereas all but 3 showed a negative IgM FA test. This finding is considered to reflect the fact that specific IgG antibodies in the serum from the patients persist for a considerably longer period of time after a *M pneumoniae* infection than the IgM antibodies (1 3)

The persistence of the IgM antibodies was examined in 9 patients in group A. The last IgM positive sample was collected up to 6 months after onset of the disease and all were found to be negative in the IgM FA test 8 to 10 months after onset of illness. *Biberfeld* (3) found that 11 out of 20 sera collected 4 years after infection yielded a positive IgM FA test with the sera diluted 1:8. The shorter persistence of the IgM antibodies observed in the present study may possibly be related to the fact that nearly all the patients had antibiotics which were effective against *M pneumoniae* during the early phase of the disease

In one patient who was suffering from pneumonia and showed a positive *M pneumoniae* CF test in a single sample, the finding of specific IgM antibodies in the FA test is compatible with a current *M pneumoniae* infection. The finding, however is not conclusive evidence of the correctness of the diagnosis since IgM antibodies were demonstrated in 3 out of 15 patients lacking clinical data compatible with a recent *M pneumoniae* infection. Most likely these IgM antibodies derived from an earlier *M pneumoniae* infection and could be detected during the course of a current virus infection. A negative IgM FA test in a single serum sample showing positive CF test tends to exclude a current *M pneumoniae* infection, especially in the younger age groups. This conclusion is valid even if the patient has been treated with antibiotics that are effective against *M pneumoniae*

The assistance of Dr *Finn Sommer* at The Department of Pediatrics, Ullevål Hospital, and the assistance received from other colleagues in collecting the samples, is gratefully acknowledged. The authors would also like to thank Miss *Turid Vollen* for excellent technical assistance.

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CELLULAR MONOSACCHARIDE PATTERNS OF *NEISSERIACEAE*

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Jantzen, E., Bryn, K. & Bøvre, K. Cellular monosaccharide patterns of *Neisseriaceae*. Acta path. microbiol. scand. Sect. B, 84 177–188, 1976.

Sixty-four strains of *Neisseria*, *Moraxella* and *Acinetobacter* were screened for cellular monosaccharides by gas-liquid chromatography and other chromatographic techniques. The four sugars ribose, glucose, glucosamine, and 2-keto-3-deoxyoctonate (KDO) were detected in all strains. Heptose was detected only in "true neisseriae" (*Neisseria gonorrhoeae*, *N meningitidis*, *N sicca*, *N cinerea*, *N flavescens*, and *N elongata*) and in the tentatively named species *Moraxella urethralis*. Some marked interspecies dissimilarities within groups were revealed. Thus, *N ovis* and *M atlantae* were characterized by the presence of mannose. Intraspecies differences were also encountered. *N meningitidis* strains of serogroups B and C were distinguished from strains of serogroup A by their sialic acid content. This sugar was also detected in two out of three examined strains of *M nonliquefaciens*. In *Acinetobacter* heterogeneity of monosaccharide patterns was rather pronounced. The results show the applicability of gas chromatographic "monosaccharide" profiles of whole cells or extracted carbohydrate in bacterial classification and identification, including differentiation at the subspecies level. In addition, such profiles may be useful for monitoring during purification of cellular polysaccharides.

Key words: *Neisseriaceae*; monosaccharide composition; gas chromatography; taxonomy

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Although carbohydrate analysis of whole cells or cell walls has proved useful in taxonomic studies of certain bacteria (8, 13 16, 24 36) the procedures have probably been considered too complicated for systematic use. We have recently described a simple gas-liquid chromatography (GLC*) technique (21) which, in addition to "finger print" and "fatty acid" profiles provides "monosaccharide" profiles† of cellular structures. The taxonomic usefulness of the two former pro-

Abbreviations:
GLC = Gas-liquid chromatography
KDO = 2-Keto-3-deoxyoctonate
LPS = Lipopolysaccharide.
MS = Mass spectrometry
TFA = Trifluoroacetyl.
TMS = Trimethylsilyl.

†The term "monosaccharide" profile refers to profiles 4, 5 and 6 of the procedure (ref. 21, Fig. 1) and includes "TFA monosaccharide", "TMS monosaccharide" and "TMS neutral monosaccharide" profiles. Monosaccharides (methylglycosides) dominate in these profiles although many other constituents also are represented (see below and ref. 21)

files for genera of *Neisseriaceae* (6 14 19 20) and *Micrococcaceae* (18) has been demonstrated. The present study comprises monosaccharide patterns of representative *Neisseriaceae* strains including those previously analysed for fatty acids. The feasibility of such monosaccharide screening for bacterial classification and identification will be discussed

MATERIALS AND METHODS

Bacterial Strains Growth and Harvesting

Forty-nine strains were studied in detail (Table 1) Forty-five of these have previously been compared by GLC (6 14 19 20) Included were also two strains of the recently described *Moraxella atlantae* A279 and 5118 (ref. 5) a serogroup C strain of *Neisseria meningitidis* (P22 cp ref. 15) and *N sicca* ATCC 9913

To investigate the differentiation between *M atlantae* and *M phenylpyruvica* the following additional strains were analysed for mannose and galactose *M phenylpyruvica* 240 A390 752/52, A1019 A1232 5542, 9158, 9413 11865/52 and *M atlantae* 8330 A1922 and B19941/75 (ref 5)

For further comparison of serogroups A, B and C of *N meningitidis* the following three strains were also examined: ATCC 13077 (serogroup A) B2515/75 (serogroup B) and B8205/75 (serogroup C) The two latter strains were recently isolated from clinical cases of meningococcal infection at the Department of Microbiology University Hospital, Tromsø, Norway

Most strains were harvested from blood agar plates after growth for 20 h at 33 C in an ordinary humid atmosphere and lyophilized (22) Cells of *N gonorrhoeae* and *N flavescens* were grown at 37 C (19)

Chemicals

Solvents of *pro analysis* grade were redistilled before use. Monosaccharides and other substances used as standards were purchased from Sigma Chemical Co. LPS preparations of *Salmonella* strains, a sample of peptidoglycan, and synthetic KDO were generously provided by Dr *O Lüderitz*, Max Planck Institut für Immunbiologie, Freiburg in Br., Germany

GLC and Peak Identification

The bacterial cells (or standards) were methanolyzed and the monosaccharide fraction TFA derivatized and freed from fatty acid methyl esters by hexane as detailed previously (21) The conditions of GLC analysis were as described (21) Major peaks were primarily characterized by retention times and co-chromatography with standards (both TFA and TMS derivatized) Some peaks

TABLE 1 *Whole Cell Monosaccharides of* Neisseriaceae

designation		Rhamnose	Ribose	Galactose	Mannose	Glucose	Heptose	Glucosamine	KDO	Sialic acid
Acinetobacter	ATCC 17903	–	1.0	0.6	–	4.2	–	5.0	0 2	–
Acinetobacter	ATCC 17978	–	1.0	1 6	–	3 1	–	3 6	0 1	–
Acinetobacter	ATCC 11171	–	1.0	0.9	–	0.7		0.9	0 1	
Acinetobacter	ATCC 17959	0.7	1 0	–	–	4.0	–	1 1	0.2	
Acinetobacter	ATCC 15149	tr	1 0	2.6	–	2.9		5.3	0.2	
Acinetobacter	ATCC 17906	–	1.0	–	–	1..		1 1	0 2	
Acinetobacter	ATCC 17977	tr	1.0	2 1	–	0.8		0 7	0 2	–
Acinetobacter	8	–	1 0	0 4	–	3.3		2.0	0.1	
Acinetobacter	9	–	1.0	1 1	–	0.8		2.6	0 1	
Acinetobacter	881/57	tr	1 0	2.2	0.9	1.6		0.3	0 2	–
Acinetobacter	2406/57	2.9	1.0	1.5	–	1.0		3.6	0 2	–
Acinetobacter	ATCC 17968	tr	1.0	1.2		1 1	–	2 4	0.1	–
Acinetobacter	ATCC 17909	2 7	1.0	.0	2 5	2.3		0.8	0 1	
Acinetobacter	ATCC 17965	–	1.0	5 0	–	6.7	–	0.4	0.2	–
Acinetobacter	ATCC 17908	0.3	1.0	3.8		1 4		5.1	0 1	–
Acinetobacter	ATCC 17924	0 4	1.0	1.2		0 9	–	1.0	0 2	–
Acinetobacter	BD4	3.2	1.0	tr	1.8	1.6	–	0.7	0 1	–
N gonorrhoeae	21319/70		1.0	1.3	–	0.6	0.8	1.9	0 6	–

TABLE 1 (cont.)

Strain designation‡		Rhamnose	Ribose	Galactose	Mannose	Glucose	Heptose	Glucosamine	KDO	Sialic acid
N. gonorrhoeae	562/71	–	1.0	0.3	–	0.2	0.2	1.4	0.5	–
N. meningitidis †	B8152/66	–	1.0	0.1	–	0.5	0.1	0.8	0.1	–
N. meningitidis§	M1	–	1.0	0.2	–	0.1	0.1	0.6	0.2	0.4
N. meningitidis⊃	P22 ep	–	1.0	0.3	–	0.4	0.3	1.2	0.1	0.7
N. sicca	ATCC 9913	0.2	1.0	0.1	–	0.3	0.2	0.8	0.1	–
N. cinerea	159/62	–	1.0	0.2	–	0.1	0.1	0.3	0.1	–
N. flavescens	ATCC 13120	–	1.0	0.1	–	0.1	0.7	0.7	0.4	–
N. flavescens	NCTC 8263	–	1.0	0.1	–	0.2	0.6	0.8	0.3	–
N. elongata	M2	0.1	1.0	0.6	–	0.1	0.7	1.0	0.7	–
N. catarrhalis	N 11	–	1.0	0.2	–	0.3	–	0.7	0.2	–
N. catarrhalis	13074/62	–	1.0	0.2	–	0.4	–	0.6	0.2	–
N. ovis	199/53	–	1.0	0.1	0.4	0.4	–	0.6	0.1	–
N. ovis	37/59	–	1.0	0.1	0.4	0.6	–	0.8	0.3	–
N. canis	ATCC 14659	–	1.0	0.2	–	0.9	–	2.1	0.2	–
N. canis	NCTC 10293	–	1.0	0.2	–	0.9	–	1.4	0.2	–
M. nonliquefaciens	4663/62	–	1.0	0.5	–	0.8	–	0.8	0.3	–
M. nonliquefaciens	NCTC 7784 N-a	–	1.0	0.2	–	0.9	–	0.8	0.3	0.5
M. nonliquefaciens	3067/66 N-a	–	1.0	0.3	–	0.7	–	0.6	0.2	0.6
M. bovis	ATCC 10900 N-a	–	1.0	0.3	–	0.8	–	1.0	0.3	–
M. bovis	4 N-b	–	1.0	0.4	–	1.2	–	1.9	0.2	–
M. lacunata	ATCC 17967		1.0	tr	–	0.1	–	0.9	0.2	–
M. lacunata	NCTC 7911	–	1.0	0.1	–	0.6	–	0.9	0.2	–
M. osloensis	A1920	1.2	1.0	1.3	–	1.3	–	1.0	0.2	–
M. osloensis	5873	tr	1.0	0.6	–	0.3	–	1.0	0.2	–
M. phenylpyruvica	2863	–	1.0	0.3	–	0.9	–	1.3	0.3	–
M. phenylpyruvica	ATCC 17958	–	1.0	0.5	–	1.1	–	1.7	0.3	–
M. atlantae	A279	–	1.0	–	5.2	0.3	–	0.4	0.2	–
M. atlantae	5118	–	1.0	–	4.9	0.7	–	0.7	0.2	–
M. kingae	4177/66 N-a	0.2	1.0	1.1	–	0.3	–	0.5	0.3	–
M. kingae	9076/70 N-a	tr	1.0	1.2	–	0.3	–	0.8	0.3	–
M. urethralis	WM6	tr	1.0	0.9	–	0.1	0.1	0.5	0.1	–

See text and ref 21 for experimental details. Identifications are primarily based on co-chromatography using standard methylglycosides (as TFA and TMS derivatives on two different gas chromatographic columns) and occasionally by GLC-MS analysis. The results were confirmed for each species by thin-layer or paper chromatography (see text). The quantitative data are approximate and to be used only for comparison. The concentrations are given relativ to the amount indicated by the second ribose peak (peak 3 Figs. 1 and 2). tr = trace amounts.

‡ N. = Neisseria. M. = Moraxella. See refs. 5, 6, 14, 15, 19, and 20 for a more complete designation of strains and their origin, characteristics, and taxonomic relations.

† Serogroup A, § serogroup B ⊃ serogroup C.

were subjected to a further characterization by GLC-MS analysis (19, 21).

The amounts of monosaccharides were estimated on the basis of the "TFA monosaccharide" profiles (Figs. 1 and 2). Peak areas were calculated by digital integrator and normalized relativ to the area of the major ribose peak (peak 3 in Figs. 1 and 2) which serves as an endogenous standard.

Carbohydrate Extraction
Thin-Layer and Paper Chromatography

10–30 mg lyophilized cells were extracted by hot aqueous phenol (33). The water phase was freed from phenol by repeated ether extractions, lyophilized, and analysed for monosaccharides either as methylglycosides by methanolysis and GLC as described above, or as free sugars by thin-layer or

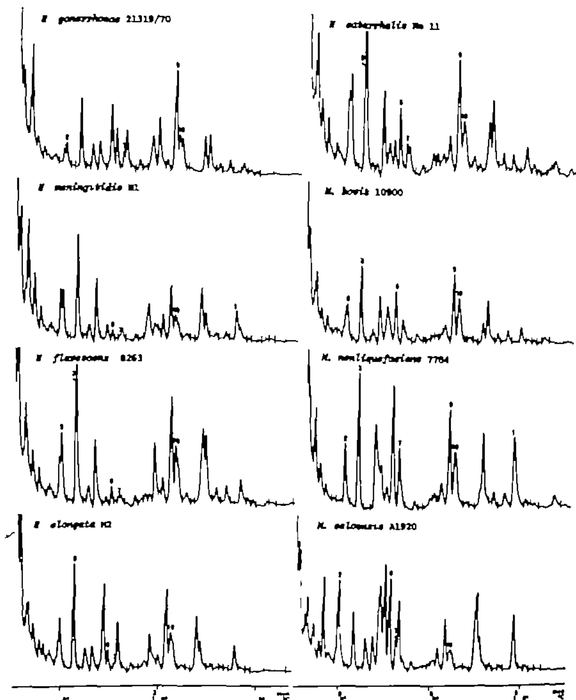


Fig 1 Representative "TFA monosaccharide" profiles of *Neisseria* and *Moraxella* species. Column: 5 per cent SP2401 on Gas-Chrom Q, 200 × 0.2 cm glass. Temperature programmed 2 C/min from 90° C to 250° C. Flow rate of carrier gas (N_2) 30 ml/min. Further chromatographic details are given in ref. 21 and in the text. *Legend* 1 rhamnose 2 and 3 ribose 4 galactose 5 mannose 6 and 7 glucose 8 heptose 9 glucosamine 10 KDO; and 11 sialic acid

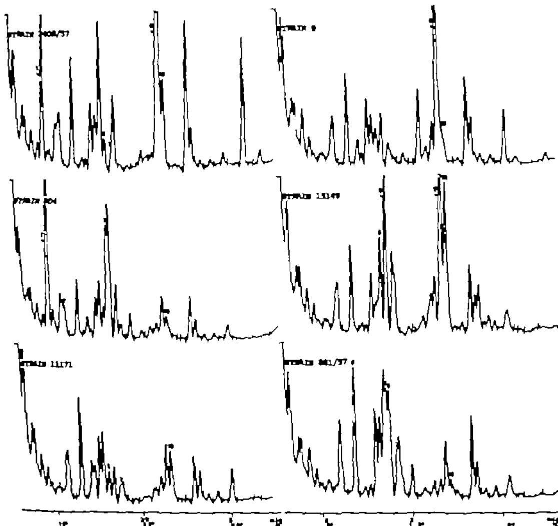


Fig. 2. Representative TFA monosaccharide profiles of *Acinetobacter* strains. See legend to Fig. 1.

paper chromatography. Free sugars were analysed as follows. The freeze-dried material was hydrolysed (2N HCl, 100° C, 4 hours) and then lyophilized (a filter of NaOH pellets adsorbed the HCl gas). The residue was dissolved in 60 per cent ethanol (10–50 µl) and applied on cellulose thin-layer plates (Macherey & Nagel Cel MN 300) or Whatman no. 1 paper. The plates and paper sheets were developed in ethylacetate/pyridine/acetic acid/water (5:3:1:3) and the components visualized by the alkaline AgNO dip reagent (34).

RESULTS

Monosaccharide Composition

As indicated in Table 1, all examined strains of *Neisseriaceae* contain ribose*, glucose, glucosamine, and KDO. Galactose is detected in all strains except *M. atlantae* and the two *Acinetobacter* strains ATCC 17959 and ATCC 17906. In general, the amounts of monosaccharides are apparently relatively high in most *Acinetobacter* strains as com-

* The enantiomeric forms (D- and L-) of the sugars have not been determined.

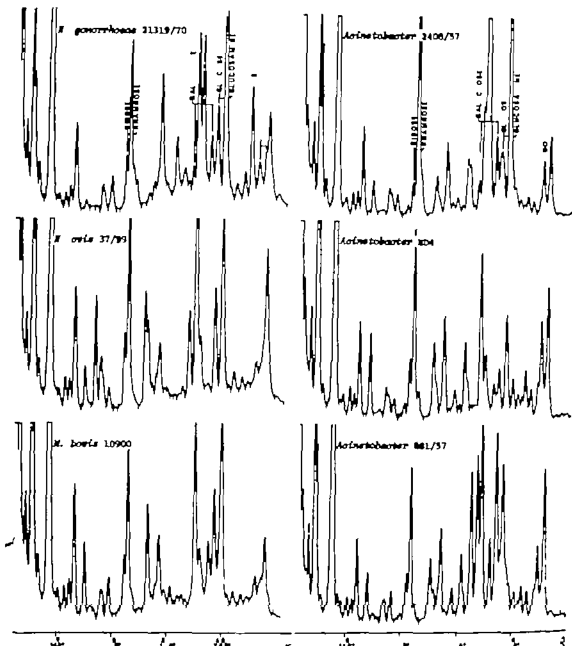


Fig. 3. Examples of "TMS monosaccharide" profiles. Column: 10 per cent W982 on Gas-Chrom Q, 200 × 0.2 cm, glass. Temperature: programmed 2° C/min from 120 °C to 260 °C. Flow rate of carrier gas (N_2): 30 ml/min. Further chromatographic details are given in ref. 21 and in the text.

pared with *Neisseria* and *Moraxella*. Heptose* is only encountered in strains of "true neisseriae" (6) and *M. urethralis* (Figs. 1, 2, and 3). *N. sicca*, *N. elongata*, *M. osloensis*, *M. kingae*, *M. urethralis* and most acinetobacters contain rhamnose, substantial amounts being encountered in *M. osloensis* A1920 and in the *Acinetobacter* strains BD1, 2108/57, ATCC 17909 and ATCC 17959. Large quan-

* The heptose peak had retention characteristics and MS fragmentation pattern identical to L-glycero-D-mannoheptose.

ities of galactose characterize the *Acinetobacter* strains ATCC 17985, ATCC 17908, and ATCC 15149. Sialic acid is present in the *N. meningitidis* serogroups B and C, but not in A (Table 1, Fig. 6). This sugar is also contained in two of the three *M. nonliquefaciens* strains examined (Table 1). Mannose apparently distinguishes *N. ovis* from other oxidase positive cocci and is present in three strains of *Acinetobacter*. In addition, mannose was detected in all strains of *M. atlantae* (the two strains of Table 1 plus strains 8530, A1922, and B19941/75), but was absent in the other oxidase positive rods, including the eleven strains of *M. phenylpyruvica* (Table 1 and Materials and Methods). The absence of galactose in *M. atlantae* (Table 1) has limited value as a marker of this species since the three additional strains of *M. atlantae* contained this sugar in small amounts, comparable with the content in a few *M. phenylpyruvica* strains.

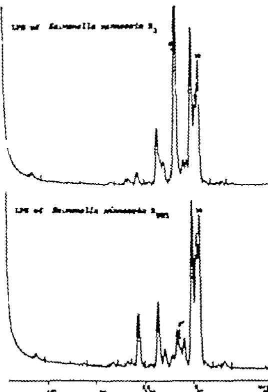


Fig. 4. "TFA monosaccharide" profiles of two LPS preparations isolated from *Salmonella minnesota* rough mutants. Lower chromatogram, "heptoseless" mutant. See text and Fig. 1 for further explanations. Retention time corresponding to the heptose peak.

Characterization of Heptose and KDO

Two typical LPS components, heptose and KDO, are recognized in the GLC "monosaccharide" profiles. Since their distribution within genera of *Neisseriaceae* has special taxonomic interest (see below), the identity of these two monosaccharides was examined more closely. Primarily their retention characteristics were found identical with those of standard LPS preparations. Fig. 4 shows "TFA monosaccharide" profiles of LPS of the *Salmonella minnesota* rough mutants R_e and R_{595} ("heptoseless"). The heptose peak in the profile of mutant R_e is easily recognized. The identity of this peak and the corresponding peaks in whole cell profiles was verified by GLC-MS analysis. Spectra obtained from *N. gonorrhoeae* 21319/70 and *N. elongata* M2 were identical to that derived from the LPS standard and to the spectrum reported by *Dass et al.* (9).

The identity of the KDO peaks were also controlled by GLC-MS analysis. Fig. 5 shows the fragmentation patterns of KDO (as TMS derivatized methylester-methylglycoside) obtained from *N. catarrhalis* Ne 11 and from the standards. A high degree of consistency is evident, particularly with the mass spectrum obtained from KDO of *S. minnesota* LPS. Identical fragmentation patterns of KDO were obtained from *N. ovis* 37/59, *N. gonorrhoeae* 21319/70, *N. elongata* M2, *M. bovis* 4 N-b, *M. lacunata* ATCC 17967, and the *Acinetobacter* strains ATCC 17977 and BD4.

The MS fragmentation patterns of KDO (Fig. 5) are typical of a TMS derivatized methylglycoside (10, 29). No molecular ion (m/e 554) is recognized, but the M-15 fragment is seen in most spectra. The highly characteristic fragments M-COOCH$_3$ (m/e 495) and M-CH$_2$OTMS (m/e 451) are easily recognized.

All mass spectra of bacterial KDO showed

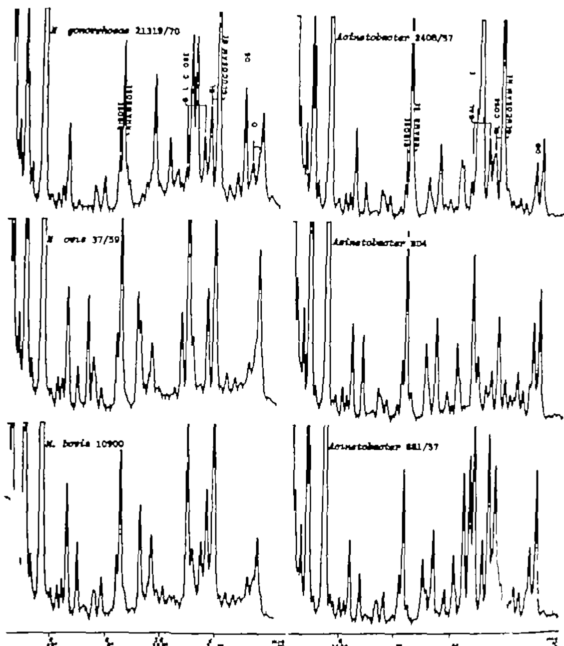


Fig 3 Examples of "TMS monosaccharide" profiles. Column 10 per cent W982 on Gas-Chrom Q, 200 × 0.2 cm, glass. Temperature programmed 2 C/min from 120 C to 260 C. Flow rat of arrier gas (N) 30 ml/min. Further chromatographic details are given in ref. 1 and in the text.

pared with *Neisseria* and *Moraxella*. Heptose* is only encountered in strains of "true neisseriae" (6) and *M urethralis* (Figs 1 2, and 3) *N sicca N elongata M osloensis M lingae M urethralis* and most acinetobacters contain rhamnose substantial amounts being encountered in *M osloensis* N1970 and in the *Acinetobacter* strains BD4 2108/57 ATCC 17909 and ATCC 17959 Large quan-

* The heptose peak had retention characteristics and MS fragmentation pattern identical to L glycero-D-mannoheptose.

tities of galactose characterize the *Acinetobacter* strains ATCC 17985 ATCC 17908, and ATCC 15149 Sialic acid is present in the *N meningitidis* serogroups B and C, but not in A (Table 1 Fig 6) This sugar is also contained in two of the three *M nonliquefaciens* strains examined (Table 1) Mannose apparently distinguishes *N ovis* from other oxidase positive cocci and is present in three strains of *Acinetobacter* In addition, mannose was detected in all strains of *M atlantae* (the two strains of Table 1 plus strains 8330 A1922 and B19941/75) but was absent in the other oxidase positive rods, including the eleven strains of *M phenylpyruvica* (Table 1 and Materials and Methods) The absence of galactose in *M atlantae* (Table 1) has limited value as a marker of this species since the three additional strains of *M atlantae* contained this sugar in small amounts, comparable with the content in a few *M phenylpyruvica* strains.

Characterization of Heptose and KDO

Two typical LPS components, heptose and KDO are recognized in the GLC "monosaccharide" profiles. Since their distribution within genera of *Neisseriaceae* has special taxonomic interest (see below) the identity of these two monosaccharides was examined more closely Primarily their retention characteristics were found identical with those of standard LPS preparations. Fig 4 shows "TFA monosaccharide" profiles of LPS of the *Salmonella minnesota* rough mutants R_5 and R_{595} ("heptoseless") The heptose peak in the profile of mutant R is easily recognized. The identity of this peak and the corresponding peaks in whole cell profiles was verified by GLC-MS analysis. Spectra obtained from *N gonorrhoeae* 21319/70 and *N elongata* M12 were identical to that derived from the LPS standard and to the spectrum reported by *Davis et al.* (9)

The identity of the KDO peaks were also controlled by GLC-MS analysis. Fig 5 shows the fragmentation patterns of KDO (as TMS derivatized methylester-methylglycoside) obtained from *N catarrhalis* Ne 11 and from the standards. A high degree of consistency is evident, particularly with the mass spectrum obtained from KDO of *S minnesota* LPS. Identical fragmentation patterns of KDO were obtained from *N ovis* 37/59 *N gonorrhoeae* 21319/70 *N elongata* M12 *M bovis* 4 N b, *M lacunata* ATCC 17967 and the *Acinetobacter* strains ATCC 17977 and BD4

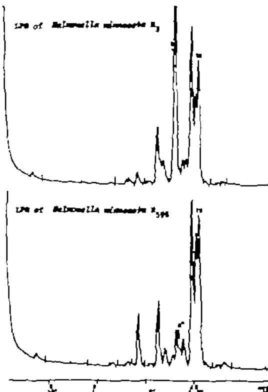


Fig 4 "TFA monosaccharide" profiles of two LPS preparations isolated from *Salmonella minnesota* rough mutants. Lower chromatogram, "heptoseless" mutant. See text and Fig. 1 for further explanations. *Retention time corresponding to the heptose peak.

The MS fragmentation patterns of KDO (Fig 5) are typical of a TMS derivative methylglycoside (10 29) No molecular ion (m/e 554) is recognized but the M-15 fragment is seen in most spectra. The characteristic fragments M-COOCH (m/e 495) and M-CH_2OTMS (m/e 4... recognized.

All mass spectra of bacteria ...

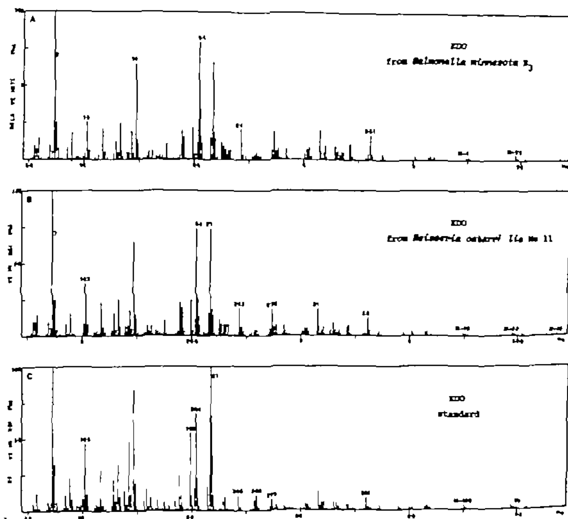


Fig 5 Mass spectrum of 2 keto-3-deoxyoctonate (KDO) as TMS *derivatized methylester methylglycoside*. Spectra obtained from, A, KDO of *Salmonella minnesota* LPS B, *Neisseria catarrhalis* N 11 whole cells and C, synthetic KDO Gas chromatography/mass spectrometry analyses were done using a 200 × 0.2 cm glass column containing 10 per cent OV 17 on 100/120 mesh Gas-Chrom Q A Varian MAT CH 7 gas chromatograph/mass spectrometer combination instrument operating at a ionizing voltage of 70 eV was used. For further details see refs. 19 and 21

some dissimilarities to the spectrum of the synthetic KDO standard (Fig. 5) with the high ratio of peak m/e 217 to m/e 204 in the spectrum of the latter as the most striking feature. These two fragments are characteristic of TMS monosaccharides in furanose and *pyranose ring forms, respectively* (29) Accordingly the GLC peak of KDO is most likely a mixture of these isomeric forms, with a higher proportion of furanose in the peak obtained from the KDO standard

Reproducibility

The influence on monosaccharide pattern of random variation in standard growth conditions was tested. Cells of *N catarrhalis* Ne 11 were harvested from nine batches of standard blood agar medium within a period of three years, six batches harvested in one laboratory and three in another No systematic differences in the nine "TTA monosaccharide" profiles could be observed. The

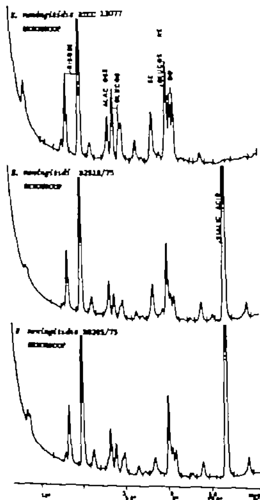


Fig. 6. "TFA monosaccharide" profiles of phenol/water extracted carbohydrates of *Neisseria meningitidis* serogroups A, B and C. See Fig. 1 for chromatographic conditions and text for further explanation.

variation in peak areas was in accordance with results previously obtained (19 22). Thus, the variation of the identified monosaccharide peaks was found to be below 15 per cent except in one case. The first heterogeneous ribose peak (peak no. 2 in Figs. 1 and 2) showed a variation of 23.2 per cent. The main ribose peak (peak 3) utilized for normalization of the chromatograms was fairly constant, the standard deviation being 10.7 per cent.

DISCUSSION

Methodological Aspects

The whole cell "monosaccharide" profiles are rather complex and the data recorded in Table 1 should be taken with a certain reservation. Thus, most peaks are evidently heterogeneous, unidentified peaks are generally present in the profiles, and the relatively high non-monosaccharide background may distort the determination of certain sugars. In addition, degradation of fragile sugars and incomplete release of others due to the high stability of certain linkages (*e.g.* phosphate esters and glycosamine bonds) may also cause inaccuracies.

In spite of such methodological difficulties, abundant compounds including the LPS sugars heptose and KDO can usually be recognized by their retention characteristics and peak configuration. Each compound, however required verification of identity and quantity. Accordingly all samples were analysed both TFA and TMS derivatized. In addition, certain identifications were controlled by mass spectrometric analysis, and thin-layer or paper chromatography was employed for each species.

Some experiments were performed in order to determine the non monosaccharide background of the profiles. Standards of protein, DNA, RNA, and peptidoglycan were subjected to the procedure. Protein and DNA did not give rise to any detectable substances, RNA yielded only ribose, whereas amino acids were released from peptidoglycan. Several unknown substances, however may also contribute to the non-monosaccharide background of the profiles.

As most monosaccharide peaks originate from extractable polysaccharides including LPS, a procedure comprising a carbohydrate extraction step prior to methanolysis was also utilized. The hot phenol method (35) gave chromatograms with considerably reduced non-monosaccharide background (Fig. 6). Of course incorporation of such an extraction step into the screening procedure will make this more laborious, but the chro-

matograms will certainly be more valuable for qualitative as well as quantitative evaluations.

The strain *N catarrhalis* Ne 11 used in the reproducibility test (see above) is apparently devoid of capsular polysaccharides, as judged by the low carbohydrate content. Owing to the well known variability of such compounds (12) the variation in monosaccharide content of strains with capsular carbohydrates can be considerably higher than that of *N catarrhalis*.

In addition to the data on carbohydrate composition, the whole cell "monosaccharide" profiles also provide considerable "finger print" information. This may be helpful as a more rapid means of differentiation of species and strains. "Monosaccharide" profiles might also be utilized for monitoring purification of bacterial wall structures. This is indicated by preliminary investigations of LPS of *Neisseriaceae* strains.

Monosaccharides of Neisseriaceae

The widely distributed monosaccharides ribose, glucose, galactose, mannose, and glucosamine were also found in the *Neisseriaceae* strains examined in the present study. Mannose differs from the others by its infrequent occurrence (Table 1). Specific LPS constituents contribute significantly to the profiles of all strains examined. In fact, the occurrence of certain LPS monosaccharides can apparently be determined directly in such whole cell "monosaccharide" profiles. Thus, KDO was recognized in all strains (Table 1). Another widely distributed LPS component, heptose, could only be detected in strains of "true neisseriae" and *M urethralis*.

Except for the deoxy sugar rhamnose other LPS constituents typical of the O-antigen part (28) such as dideoxy sugars, have not been recognized in the profiles. The relatively small amounts of rhamnose found in *N elongata* originates from LPS (unpublished results) but the same seems not to apply to the large amounts of rhamnose in the type strain of *M osloensis* (A1920) and some of the acinetobacters. A capsular rhamnose-glucose polysaccharide in *Acinetobacter calcoaceticus* BD4 has been reported (32). Similar polysaccharides may occur in the other strains with a high content of monosaccharides (Table 1) as *Acinetobacter* appears to be frequently encapsulated (30).

The meningococcal serogroup A capsular antigen has been shown to be essentially a homopolymer of O-acetylated 2-acetamido-2-deoxymannosyl phosphate (26). However mannosamine was not detected in the profiles of the group A strains, probably because of the high stability of the phosphodiester bonds of this polysaccharide. Mannosamine may not be liberated under conditions as those in the present investigation. Serogroups B and C polysaccharides are also homopolymeric carbohydrates but with sialic acid as monomeric unit (25). Accordingly sialic acid was detected in the strains of groups B and C (Table 1 and Fig 6). The presence of sialic acid in two of the *M nonliquefaciens* strains is interesting and suggests further studies.

Taxonomic Implications

The genetic relations and systematics of most organisms examined here have been discussed in connection with the results of fatty acid analyses (5 6 14 19 20). As regards their monosaccharide composition, the distribution of the specific LPS constituents KDO and heptose may be of particular taxonomic interest.

KDO has been found in almost all Gram-negative bacteria hitherto studied (11 27). The present results show that the family *Neisseriaceae* does not represent an exception. This finding is in agreement with previous studies of *N meningitidis* (23), *N gonorrhoeae* (31) and of an *Acinetobacter* strain (33) while it is in contrast to the results obtained by *Adams et al* (3 4). They did not detect KDO in LPS of *N catarrhalis* ATCC 8176, *M duplex* var *nonliquefaciens* ATCC 19955 (presently named *Moraxella lacunata* (17)) and *Micrococcus calcoaceticus* ATCC 23055 (presently named *Acinetobacter calcoaceticus* (17)).

The detection of heptose in "true neisseriae" is in accordance with previous LPS analyses (2, 23 31) However N sicca also a "true neisseriae" species (7) has been reported to lack heptose (1) which is in contrast to the results obtained in the present study (Table 1) Our results indicate that heptose generally is present in "true neisseriae" (including the rodshaped *N elongata*) and absent in "false neisseriae" *Moraxella* (except the tentatively named *M urethralis*) and *Acinetobacter* This difference is in agreement with the lack of genetic affiliation between "true neisseriae" and other groups of *Neisseriaceae* and demonstrates the value of monosaccharide analysis for classification.

Except for the distribution of heptose, the "monosaccharide" profiles do not distinguish equally clearly between the main groups of *Neisseriaceae* as the fatty acid composition (19 20) However specific carbohydrate patterns can be used for species identification in selected cases as exemplified by the distinction between *M phenylpyruvica* and *M atlantae* which are of a similar fatty acid composition (5) Furthermore, "monosaccharide" profiles seem to be a potential tool in subspecific differentiation.

The mass spectral analyses were performed at the Institute of Clinical Biochemistry University of Oslo, Rikshospitalet, Oslo. The valuable assistance of Dr *E. Jellum* and M *P Helland* of that institute is gratefully acknowledged. W also want to thank Dr *O Lüderitz*, Max Planck Institut für Immunbiologie Freiberg in Br Germany for the gift of peptidoglycan, lipopolysaccharides, and synthetic KDO

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THE EFFECT OF ENDO-ENTERIC DEVELOPMENT OF *TOXOPLASMA GONDII* ON THE ULTRASTRUCTURE OF EPITHELIAL CELLS OF THE SMALL INTESTINE OF INFECTED CATS

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Ferguson, D. J. P., Hutchison, W. M. & Siim, J. Chr. The effect of endo-enteric development of *Toxoplasma gondii* on the ultrastructure of epithelial cells of the small intestine of infected cats. Acta path. microbiol. scand. Sect. B 84 189–195 1976.

Toxoplasma gondii undergoing endo-enteric development in the small intestine of the cat affects the ultrastructure of the epithelial cells. The epithelial cells from infected cats have swollen rough endoplasmic reticulum and the mitochondria may be swollen with degenerate cristae. A statistically significant shortening of the microvilli of the epithelial cells was demonstrated. It appeared that the effect on the microvilli length was related to the parasite distribution within the small intestine. These changes occur in both cells with and without parasites present at the level of the sections studied. The abnormalities are quickly rectified after the disappearance of the endo-enteric forms of *Toxoplasma*.

Key words: *Toxoplasma gondii*; endo-enteric development; epithelial cells of the small intestine; ultrastructure; cats.

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The discovery of the coccidian life cycle of *Toxoplasma gondii* in the epithelial cells of the small intestine of the cat was reported by *Hutchison et al.* (1969 1970) *Sheffield & Melton* (1970) *Frenkel et al* (1970) *Pickarski & Witte* (1970) and *Overdulve* (1970). The oocyst, produced by this development sporulates to form two sporocysts each containing four sporozoites (*Siim et al.* 1969) and thus has a structure similar to that of members of the genus *Isospora*.

In light microscope studies of Coccidia belonging to *Isospora* and the closely related genus *Eimeria* it was observed that these infections caused changes in the structure of the villi of the intestine of the host (*Pout* 1967 a & b *French et al* 1964 *Brandborg et al* 1970 *Fernando & McCraw* 1973). In a short note, *Sheppard* (1974) described the ultrastructural changes which had occurred

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in the epithelial cells of chickens infected with *Eimeria necatrix*.

This paper reports the effect of the endo-enteric development of *Toxoplasma gondii* on the ultrastructure of the epithelial cells of the small intestine of the cat.

MATERIALS AND METHODS

Nine Specific Pathogen Free (S.P.F.) cats were used in this study. They were divided randomly into three groups each of three cats: the first was examined as uninfected controls, the second was infected, and the cats were killed 7–10 days post infection while still producing faeces containing oocysts, and the third was infected and produced faeces containing oocysts, but the cats were not killed until 7 days after the oocysts had disappeared from their faeces. This group will be referred to as "recovered" cats. That is, they had recovered from the endo-enteric development although exo-enteric development might still have been taking place.

The infected and recovered cats were infected as previously described (*Hutchison et al.* 1971).

All cats were tested serologically prior to the experiment and were Dye Test negative, but, whereas the control cats remained negative, the infected and "recovered" cats were found to be Dye Test positive at the time of autopsy.

The same procedure was used with all cats at autopsy. The small intestine was removed and divided into 24 equal portions. Samples from five portions of the small intestine as indicated in Fig. 1 were treated for light and electron microscopic examination. Histological examination was carried out as described by *Hutchison et al.* (1971). For electron microscopy samples were treated as described previously by *Ferguson et al.* (1975).

RESULTS

Cytoplasmic Changes

When the epithelial cells from the control cats were examined they were found to be columnar shaped with a brush border of microvilli protruding into the lumen of the intestine. Below the microvilli was an area of cytoplasm which was densely packed with filaments: these filaments formed the terminal web. The cytoplasm contained rough endoplasmic reticulum, Golgi apparatus, mitochondria with plate-like cristae and a nucleus possessing one or more nucleoli (Figs. 2 & 5).

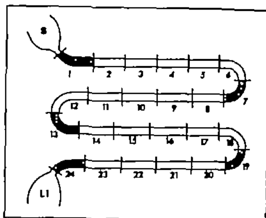


Fig. 1 A diagram showing the division of the small intestine into 24 equal portions. The five portions examined in this study were Numbers 1, 7, 13, 19 and 24 (the shaded areas). S Stomach, LI Large Intestine.

In the epithelial cells from infected cats, similar organelles were present but in this case the cytoplasm appeared vacuolated (Fig. 3). At higher magnification the vacuolisation appeared to be due to a swelling of the cisternae of rough endoplasmic reticulum (Fig. 6). The mitochondria were swollen and had degenerate cristae (Fig. 6). The changes described occurred whether the cells in the sections examined contained developing toxoplasms or not.

The epithelial cells of the "recovered" cats were similar to those of the controls with normal appearance of the rough endoplasmic reticulum and of the mitochondria (Figs. 4 & 7).

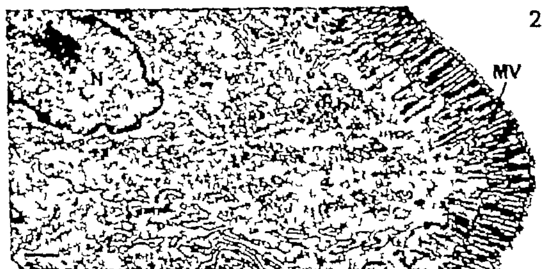
Figs. 2–10 are, in all cases, micrographs of sections of epithelial cells taken near the tips of villi from Portion 19 of the small intestine of the three groups of cats. The bar on each micrograph represents 1 µm.

Figs. 2–4 Epithelial cells showing the microvilli, terminal web, rough endoplasmic reticulum, Golgi apparatus, mitochondria and nuclei.

Fig. 2 From a control cat. × 10,000.

Fig. 3 From an infected cat. × 10,000.

Fig. 4 From a "recovered" cat. × 10 000.


2
N
MV


3
MV
N


4
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MV

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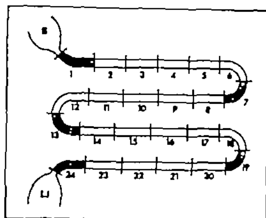


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Fig. From a control cat. × 10,000.

Fig. 3 From an infected cat. × 10,000.

Fig. 4 From a "recovered" cat. × 10,000.

TABLE 1 *The Average Length of the Microvilli in Control, Infected and "Recovered" Cats and the Parasite Distribution within the Infected Group*

Portion of the small intestine	Length of the microvilli/µm			Infected group Average No. of parasites/4 µm villus section
	Control	Infected	"Recovered"	
1	1.44 ± 0.01	1.22 ± 0.27	1.49 ± 0.10	3.4
7	1.50 ± 0.11	0.88 ± 0.18	1.27 ± 0.21	8.1
15	1.16 ± 0.06	0.75 ± 0.10	1.06 ± 0.12	17.3
19	1.04 ± 0.10	0.50 ± 0.03	1.17 ± 0.13	20.2
24	1.08 ± 0.15	0.63 ± 0.04	1.00 ± 0.15	19.8
average	1.25 ± 0.12	0.78 ± 0.04	1.25 ± 0.19	

The length and standard deviation of the microvilli were calculated from the average length found for each of the members of the group. This average length for the individual cats was based on ten measurements.

Changes in Microvilli Lengths

The typical appearance of microvilli from Portion 19 (as defined in Fig 1) of the small intestine of the three groups of cats is shown in Figs. 8, 9 & 10. The microvilli of the cells of the infected group appear shorter than those of the control and "recovered" groups. In all cases, the region of the tip of a villus was examined. The average length and standard deviation within the three members of each group were calculated for each of the five portions of the small intestine, and the results are given in Table 1 The variation in length of the microvilli was small within the members of each group The table shows that for the control group there was a slight reduction in the length of the microvilli from Portion 1 to Portion 24 (Fig 1) of the small intestine.

The results for the "recovered" cats were similar to those of the control group, Table 1 When the average length of the microvilli for these two groups were compared statistically using the Student "t" Test, no significant difference was found ($P>0.50$)

In the infected group, there was a marked reduction in the length of the microvilli. When the average length of the microvilli of this group was compared to the control and "recovered" groups, it was found that there was a significant difference ($P<0.001$)

Sections from the infected group of three cats were examined by light microscopy and the number of intracellular parasites per 4 µm section of villi was counted. The average number for each portion is given in Table 1 It was found that the number of

Fig 5 Cytoplasm of an epithelial cell from a control cat showing the mitochondria, Golgi apparatus, and rough endoplasmic reticulum. × 30,000.

Fig 6 Cytoplasm of an epithelial cell from an infected cat showing the Golgi apparatus, some swollen mitochondria, and swollen rough endoplasmic reticulum. × 30,000.

Fig 7 Cytoplasm of an epithelial cell from a "recovered" cat showing normal mitochondria and rough endoplasmic reticulum. × 30 000

Fig 8. Detail (cf Fig. 2) showing the microvilli from a control cat. × 30,000.

Fig 9 Detail (cf Fig 3) showing the stunted appearance of microvilli from an infected cat. × 30,000.

Fig 10 Detail (cf Fig 4) showing the microvilli from a "recovered" cat. × 30,000.

Abbreviations in the electron micrographs
ER: Rough Endoplasmic Reticulum
G: Golgi Apparatus
Mi: Mitochondrion
MV: Microvilli
N: Nucleus
TW: Terminal Web

parasites increased towards the posterior end of the small intestine. From Table 1 it can also be seen that the reduction in length of the microvilli is most marked in the areas containing the largest numbers of parasites. It would thus appear that the effect on the microvilli is related to parasite distribution within the epithelial cells of the different regions of the small intestine.

DISCUSSION

The effects observed in this study can be directly attributed to *Toxoplasma* infection since S.P.F. cats were used. Further evidence for this was the fact that the cat villi returned to normal after the disappearance of the endo-enteric forms of *Toxoplasma*.

It was previously observed that in the infected cats the villi were shorter and the crypts of Lieberkühn deeper (*Bain & Hutchison* unpublished). Similar changes were noted in the present study and they were most marked in the areas in which the microvilli were shortest. It would thus appear that the changes in microvilli length parallel those of the villi. These results on the effect on the villi length were similar to those reported for *Eimeria* and *Isospora* infections (*Pout* 1967 a & b, *Fernando & McCraw* 1973 *French et al.* 1964 *Brandborg et al.* 1970).

The ultrastructural changes in the host cell cytoplasm which were observed in infected cats (i.e. swollen endoplasmic reticulum and mitochondria) were similar to well known fixation artifacts. However it was not believed that the changes noted were preparation artifacts, since control and "recovered" cats, treated in exactly the same manner did not exhibit these changes. Moreover the changes were similar to the cytoplasmic changes reported in *Eimeria nieschulzi* infections (*Sheppard* 1974).

The shortening of the microvilli is apparently directly proportional to the number of parasites present within the villi. This is similar to the effect on villi length previously noted (*Bain & Hutchison* unpublished). The microvilli are also shortened in *Eimeria nieschulzi* infections (*Sheppard* 1974). This shortening of the microvilli has been reported in certain human malabsorptive disorders by *Curran & Creamer* (1963) and *Trier et al.* (1965). With *Eimeria nieschulzi* these ultrastructural changes were occurring at the time of reduced glucose absorption (*Sheppard* 1974). The structure and function of the intestine are thought to be closely interrelated (*Trier* 1967, *Padykula* 1962). It would therefore be expected that these morphological changes in the epithelial cells in cats infected with *Toxoplasma* are affecting the efficiency of the intestine.

The morphological changes were occurring in all epithelial cells whether the cells within the sections studied contained parasites or not. This is similar to *Eimeria nieschulzi* infections (*Sheppard* 1974). It is possible that a coccidian-produced toxic factor could cause this general effect. However similar changes have also been reported in nippostrongylosis by *Symons et al.* (1971), in bovine ostertagiasis by *Anderson et al.* (1965) and in some non-parasitic diseases by *Curran & Creamer* (1963). From this, it would appear that the changes reported represent a general reaction of the intestine to many disorders and represent a sign of malabsorption.

The almost complete return to normal of the microvilli and cytoplasmic organelles of the epithelial cells after the disappearance of the endo-enteric forms of the parasite is similar to that reported in the treatment of some non-parasitic disorders in man (*Greenwald et al.* 1975).

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THE EFFECT OF GLUTARALDEHYDE ON THE STABILITY OF ERYTHROCYTES AND ON VIRUS RECEPTOR SUBSTANCES

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Dalen, A. B. The effect of glutaraldehyde on the stability of erythrocytes and on virus receptor substances. Acta path. microbiol. scand. Sect. B 84 196–200 1976

Fixation with glutaraldehyde at a concentration above 0.05 per cent for 1 h at neutrality rendered erythrocytes resistant towards surfactants as Triton X 100, Tween-80 and sodium dodecyl sulphate. Fixation of erythrocytes with low concentrations of glutaraldehyde abolished the lytic effect of membrane active proteins as complement, phospholipase C and staphylococcal α-toxin. The fixation procedure did not alter significantly the receptor groups on erythocytes for viruses of the ortho- and para-myxo group and rubella virus. The fixation reduced the agglutinability of human O-erythrocytes by reovirus.

Key words Erythroyctes virus receptors stability glutaraldehyde.

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Glutaraldehyde has been widely used as a fixative of cells to be examined in the electron microscope. It has been shown that various enzymes retain their activity after conjugation with glutaraldehyde (1) Immunoglobulins retain their biological activity after treatment with glutaraldehyde and glutaraldehyde has been used for preparation of insoluble immunoadsorbents (2) Glutaraldehyde has also been used as a coupling *reagent* in passive haemagglutination tests (3) Protein antigens were effectively bound to the erythrocytes when they contained a relatively high number of free amino groups. The cells coated with the aid of glutaraldehyde could be lyophilized without loss of agglutination titre.

The present work is related to the use of glutaraldehyde as a *coupling reagent* in virus serology The stability of glutaraldehyde-treated erythrocytes towards various surface active substances and the effect of glutaraldehyde on certain receptor substances of viruses are reported

MATERIALS AND METHODS

Erythrocytes

Erythrocytes of various animal species were obtained from whole blood collected in Alsever's solution Human O Rh-erythrocytes were obtained *from outdated bank blood stored in sterile* citrate-dextrose solution for 4 5 weeks Before use the erythrocytes were washed four times in 6 10 volumes of phosphate buffered saline (PBS) pH 7.2. They were finally packed at 1,000 × *g* for 5 min

Virus

Newcastle Disease Virus (NDV) influenza A virus (strain PR8 HON1) and parainfluenza virus I (Sendai virus) were propagated by inoculation

into the chick allantoic sack of 10-day-old embryonated eggs. Allantoic fluid was harvested after 2 days of inoculation and used as a source of haemagglutinin.

Reovirus (serotype 1) was propagated in Vero cell monolayers, grown in Eagle's Minimum Essential Medium supplemented with 5 per cent inactivated foetal calf serum. Cells were harvested when the culture showed widespread cytopathogenic changes. The cells were collected by centrifugation, frozen and thawed three times and used as a source of haemagglutinin.

Commercial preparations of mumps haemagglutinin (Orion Oy Helsinki Finland) and rubella haemagglutinin (Behringwerke, Mannheim, W Germany) were used.

Fixation of Erythrocytes

Varying quantities of glutaraldehyde (Serva, Heidelberg, W Germany 25 per cent aqueous solution) were added to a 1 per cent, thrice-washed erythrocyte suspension in PBS The suspension was gently stirred for 1 h at room temperature and washed three times in PBS.

Agglutination Test

This was performed in Linbro plastic trays. A serial twofold dilution of the virus haemagglutinin suspension was made in various diluents. The dextrose-gelatin-veronal (DGV) buffer (5) used had a pH of 7.3 and the following composition (in 1000 ml) veronal: 0.58 g, sodium veronal: 0.38 g, gelatin 0.60 g, $CaCl_2$ (anhydrous): 0.02 g, $MgSO_4 \cdot 7H_2O$ 0.12 g, NaCl: 8.50 g and dextrose 10.00 g.

To each dilution was added 0.05 ml of an 0.25 per cent erythrocyte suspension. The pattern of haemagglutination was read when evident, usually after 1 h of incubation at room temperature.

Lytic Assay

Sodium dodecyl sulphate (SDS) Triton X 100 and Tween-80 were analytical grade reagents. A serial twofold dilution of each surfactant was made in Linbro plastic trays using PBS as the diluent. To each dilution was added an equal volume (0.025 ml) of 1 per cent erythrocyte suspension in PBS. After incubation at 37 C for 30 min, haemolysis was determined visually and the concentration of the surfactant giving a 50 per cent haemolysis was recorded.

Pure staphylococcal α-toxin was obtained by electrophoresis at its iso-electric point (4) The specific activity was 25,000 haemolytic units per mg protein and the concentration 13,000 haemolytic units per ml Haemolytic activity was titrated by making serial twofold dilutions in tubes with PBS as the diluent. An equal volume (0.5 ml) of 1 per cent rabbit erythrocyte suspension in PBS was added to each dilution. Incubation was performed at 37 C for 30 min and one haemolytic unit was defined as the amount giving a 50 per cent haemolysis of the erythrocyte suspension.

Phospholipase C (ex *Clostridium perfringens*) was obtained from Koch-Light (Colnbrook, Bucks, England) A serial twofold dilution in tubes was performed in phosphate buffered saline (pH 6.2) with $CaCl_2$ (10^{-3} M) An equal volume (0.5 ml) of a 1 per cent suspension of the various erythrocytes to be tested was added. The dilution giving a 50 per cent haemolysis was determined visually after incubation at 37 C for 30 min.

RESULTS

The Stability and the Effect of Lytic Proteins on Glutaraldehyde-treated Erythrocytes

Erythrocytes from sheep guinea-pigs, rabbits, hens and man were treated for 1 h with glutaraldehyde in concentrations of 0.1 0.05 0.025 and 0.0125 per cent in PBS. The stability of the erythrocytes during storage was examined by keeping a 1 per cent solution of the erythrocytes in PBS at 4 C. Haemolysis was obvious after about 1 week in all types of erythrocytes being fixed by the lowest concentration of glutaraldehyde (0.0125 per cent) while the erythrocytes treated with 0.1 per cent appeared undamaged even after several weeks of storage.

The effect of phospholipase C was tested on fixed erythrocytes from man, hens and sheep. Fixation with glutaraldehyde, 0.0125 per cent final concentration, increased the resistance of the erythrocytes at least 1,000 fold. A haemagglutination pattern was produced by the enzyme at a titre which corresponded to the haemolytic titre of non-fixed erythrocytes. No significant difference in behaviour of the three species was observed.

Rabbit erythrocytes, which are particularly sensitive to the haemolytic action of staphylococcal α-toxin, were rendered resistant to the toxin by fixation in glutaraldehyde at a concentration of 0.0125 per cent. The activity of the highest concentration of toxin tested was 13,000 haemolytic units per ml.

The effect of complement was examined with sheep erythrocytes fixed with glutaraldehyde in concentrations from 0.0125 to

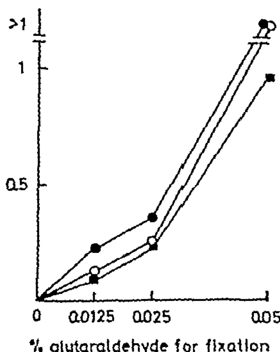


Fig 1 The stability of erythrocytes fixed with various concentrations of glutaraldehyde against the lytic effect of sodium dodecyl sulphate (SDS) —■— rabbit erythrocytes, —○— human erythrocytes, —●— hen erythrocytes.

0.1 per cent. The erythrocytes were completely resistant while the agglutination titre of rabbit anti-sheep erythrocytes globulin remained unchanged after treatment with glutaraldehyde.

The Effect of Surfactants on Glutaraldehyde-treated Erythrocytes

Erythrocytes (0.5 per cent) from sheep, man, rabbits and guinea-pigs were lysed in Tween-80 (0.25 to 0.5 per cent) Triton X 100 (0.06 per cent) and sodium dodecyl sulphate (0.003 to 0.012 per cent) The species differences in sensitivity were small, hen erythrocytes being generally the most resistant and rabbit erythrocytes the most sensitive to the treatment. Glutaraldehyde at final concentrations of 0.0125 to 0.1 per cent was used for fixation under standard conditions. Fixation increased the resistance to lysis considerably as seen in Fig 1 which illustrates the effect of SDS on treated and untreated erythrocytes from rabbit, hen and man. A similar increase in resistance after glutaraldehyde treatment with Triton X 100 was seen.

Human erythrocytes fixed with glutaraldehyde (0.1 per cent final concentration) were exposed to a lipid extraction procedure Tween-80 at a 1 per cent solution, was added slowly to a final concentration of 0.1 per cent, and ½ volume of anaesthetic ether was added. After stirring for 1 h the two phases were separated A considerable amount of material accumulated in the ether layer The erythrocytes in the aqueous layer appeared normal on microscopy and they settled as a button on titration in plastic trays.

The Effect of Glutaraldehyde on Erythrocyte Virus Receptors

Comparison of haemagglutinating titres of various viruses within the myxo-virus group on erythrocytes fixed with different concentrations of glutaraldehyde revealed slight differences. The erythrocytes were fixed with glutaraldehyde one week prior to the titration and stored at +4 C. The drop in sensitivity to be seen if hen blood were treated with the lowest concentration of glutaraldehyde (0.0125 per cent) probably was related to a beginning haemolysis Fig 2) This was not seen in the case of guinea-pig cells.

Reo-virus 1 which binds to an unknown receptor on human erythrocytes, behaved differently Increasing concentrations of glutaraldehyde reduced the sensitivity of the erythrocytes to virus haemagglutination (Fig 3)

The sensitivity of sheep and hen erythrocytes towards rubella haemagglutinin was not significantly altered (Fig. 4) and the haemagglutination pattern was easy to read. Hen erythrocytes had to be treated while they were in a fresh state otherwise a drop in sensitivity was seen. This seemed less important in the case of sheep erythrocytes.

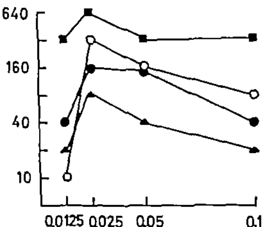


Fig 2 Effect of glutaraldehyde on the sensitivity of hen erythrocytes to virus haemagglutinins.
—▲— Newcastle Disease Virus,
—■— Influenza virus A (PR8 HON 1)
—○— Para-influenza virus I (Sendai)
—●— Parotitis irus.

Choice of Buffer

Treatment with glutaraldehyde increased the capacity of the erythrocytes to adhere to the plastic wall when PBS was used as a diluent. Tris-chloride buffer (0 05 M pH 7.5) rendered isotonic with NaCl behaved in a similar manner Addition of versene (0 0125–0 1 M) enhanced the stickiness. Heparin (50 I.U) Mg^{+} and Ca^{2+} (10^{-3} M) were all without influence on the adherence.

Hypotonic phosphate buffer (0 05 M, pH 7.2) and tris-chloride buffer (0.05 M, pH 7.5) were tried as diluents. If compared with the corresponding buffers rendered isotonic by addition of NaCl, no difference in rate of sedimentation or pattern would be observed.

Addition of Tween-80 (final concentration 0 025 per cent) or bovine serum albumin (1 per cent) reduced the adhesion. Dextrose gelatin veronal buffer however generally gave the most distinct pattern of the virus haemagglutination reactions. This was also the case if the various virus haemagglutinins were tested in serial dilutions of antibody negative sera.

DISCUSSION

Cross-linking of proteins with glutaraldehyde requires the presence of free amino groups

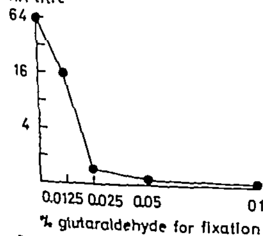


Fig 3 Effect of glutaraldehyde on the sensitivity of human erythrocytes for haemagglutination with reovirus I

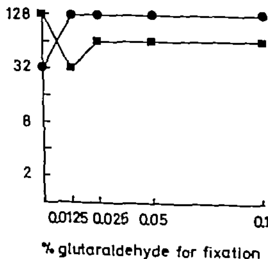


Fig 4 Effect of glutaraldehyde on the sensitivity of sheep erythrocytes (■—■) and hen erythrocytes (●—●) for rubella haemagglutinin.

(2) In proteins, the free epsilon amino groups of lysine probably are the most important in the reaction. Other reactivities of glutaraldehyde would seem probable, but are unknown. In the case of proteins, a minimal critical number of free amino groups has to be present, otherwise no cross-linking takes place. Glutaraldehyde has many advantages as a coupling reagent of infectious material to erythrocytes for serological use. The antigens remain intact to a large extent, and the cells can be stored for very long periods of time. Furthermore, glutaraldehyde has a well-documented disinfectant activity on viruses (8) The stabilizing effect of erythrocytes is probably due to cross-linking of the membrane proteins. The extraction of lipids from fixed erythrocytes under rather harsh conditions did not cause break-down of the cells. The increased resistance of the erythrocyte membranes against proteins such as staphylococcal α toxin and phospholipase C caused by glutaraldehyde was far higher than the general resistance towards the low molecular surfactants. This might be due to a specific effect of the two proteins on the substrates. The substrate for staphylococcal α toxin in the erythrocyte membranes is unknown and an enzymatic nature of its effect has not been established Using a concentration of glutaraldehyde of about 0.05 per cent to a 1 per cent erythrocyte solution, erythrocytes are obtained which can be freeze-dried and reconstituted, kept in distilled water and exposed to various strong membrane-active substances.

The receptor group of influenza virus and the viruses of the paramyxo group Newcastle Disease Virus, para influenza virus and parotitis virus, is N-acetyl-neuraminic acid located on the surface of various erythrocytes. Nothing seemed to suggest that glutaraldehyde affected the receptor groups to any significant extent with any of the erythrocytes tested.

The receptor group of reovirus is unknown, but trypsin reduces the agglutinability of human O-erythrocytes (6) The main reactive site on the red cells surface is therefore thought to be a protein. The observed reduction in agglutinability of human O-erythrocytes after glutaraldehyde fixation could be caused by an inhibited access of the virus to the receptor group rather than a destruction of the receptor group. It has been shown *that glutaraldehyde binds strongly to the* surface layers of *E coli* and probably prevents the access of low-molecular weight substrates to membrane-bound enzymes (7)

Little is known about the receptor of rubella viruses, but evidence suggests that it is of lipid nature. Fixation with glutaraldehyde did not reduce the sensitivity to the rubella haemagglutinin and the treated cells could be stored in suspension without any loss of sensitivity as otherwise seen in the case of untreated cells.

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AMPICILLIN SENSITIVITY AND BIOTYPES OF RECENT DANISH ISOLATES OF *HAEMOPHILUS INFLUENZAE*

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Bruun, B. & Friis-Møller A. Ampicillin sensitivity and biotypes of recent Danish isolates of *Haemophilus influenzae*. Acta path. microbiol. scand. Sect. B, 84 201–204 1976.

Among 148 strains of *Haemophilus influenzae* isolated in 1975 from different localities in Denmark, none was found to be resistant to ampicillin. A majority of the strains belonged to biotypes I II and III and the presence of a partial correlation between biotype and origin was confirmed.

Key words *Haemophilus influenzae* ampicillin sensitivity biotypes Danish isolates.

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Since 1970 an increasing number of reports on *Haemophilus influenzae* strains resistant to ampicillin have been published in the U.S., Great Britain and Germany (6, 8, 13) As ampicillin for some time has been the drug of choice in the treatment of *H influenzae* infections, we considered it of importance to find out whether resistant strains occurred also in Denmark.

At the same time we found it of interest to see whether the distribution among the biotypes of *H influenzae* and the correlation between biotypes and sources of strains, as found by *Kilian* (5) would hold true for a collection of Danish *H influenzae* strains recently isolated.

MATERIAL AND METHODS

One hundred seventy-six strains received as *H influenzae* from bacteriological laboratories in Denmark during July to October 1975 were examined. The strains were isolated from various infections including meningitis, bacteraemia, otitis and respiratory diseases, but strains from the respiratory tract predominated (Table 1) Because of lack of clinical information it has not been possible to distinguish between respiratory saprophytes and respiratory pathogens, but it is considered likely that a majority are saprophytes which probably form part of the normal flora. The criteria on which a strain is included in the survey as a strain of *H influenzae* were: a negative Gram stain, coccobacillary or rod-shaped cells, a requirement for the two growth factors haemin and nicotineamide adenine dinucleotide (NAD) (or one of its precursors (10)) and lack of haemolysis on horse blood agar When these criteria were applied, 148 of the 176 strains were identified as *H influenzae* and thus included in the survey the remaining strains mostly belonged to the species *H parainfluenzae*

Two strains of ampicillin resistant *H influenzae* one of which was a serotype b, were provided by the Center for Disease Control, U.S. Department of Health, Education and Welfare Atlanta, Georgia they were used as reference strains for the production of β-lactamase.

Culture Methods

Chocolate agar (1.5 per cent Orthana peptone 0.1 per cent Oxoid yeast extract, 0.5 per cent NaCl, 0.015 per cent citric acid, 0.18 per cent K_2HPO 0.2 per cent Merck starch no. 1252, 0.8 per cent Japan agar 2 per cent liver autolysate, 0.15 per cent glucose, 7 per cent heat treated blood) was used for maintaining the strains. The plates were incubated at 37 C in an atmosphere containing approximately 10 per cent CO_2.

Levinthal broth (1) was used for fluid cultures. Levinthal agar (1) was used for the detection of β-lactamase.

Morphology and Staining

A saline suspension of a 24-hour colony from chocolate agar was examined by phase contrast microscopy for shape and motility and a heat fixed film was Gram stained.

Biochemical Tests

a) Growth factor requirements The requirement for haemin which for practical purposes may be equated with the lack of ability to synthesize porphyrins from δ-aminolaevulinic acid, was determined by the porphyrin test described by *Kilian* (4) The requirement for NAD was tested by observing the satellite phenomenon around a colony of *Staphylococcus albus* on 5 per cent horse blood agar

b) Haemolysis was looked for after growth on 5 per cent horse blood agar for 24 hours.

c) Indole production was demonstrated in a 48-hour Levinthal broth culture, using the Ehrlich-Böhme reagent.

d) Urease activity was demonstrated as described by *Jessen* (2)

e) Ornithine decarboxylase was demonstrated as described by *Møller* (7)

f) Production of acid from carbohydrates was determined in phenol red broth base (Difco) The carbohydrates were added to the autoclaved medium to give a final concentration of 1 per cent (w/v) Haemin chloride (Fluka) and nicotineamide adenine dinucleotide (Merck) was added in filter sterilized solutions to a final concentration of 10 μg/ml. The media were inoculated with bacteria from 24-hour chocolate plates and incubated for 5 days at 35 C (without addition of CO_2 to the atmosphere) The following carbohydrates were used D(+)-glucose sucrose, lactose D(+) xylose and D(—)-mannitol. An inoculated control of basal medium was included for each strain to detect any increase or decrease in pH of non-glycolytic origin.

Serology

The possession of a type b capsule was determined by the capsule swelling technique using antiserum b, produced by the Pneumococcus Department, Statens Seruminstitut, and colonies from a 24-hour chocolate agar plate.

Ampicillin Susceptibility

Tests for ampicillin susceptibility were done by the prediffusion disc method described by *Thomsen* (12) and by demonstration of production of the β-lactamase which has been shown to be the enzyme causing ampicillin resistance in *H influenzae* strains (3 11)

For the prediffusion method, discs containing 15 μg ampicillin and the special antibiotic testing agar (0.35 per cent KCl, 0.8 per cent Na_2HPO 12 H_2O 0.25 per cent Sheffield Chemicals NZ-amine type B 0 125 per cent Orthana yeast hydrolysate 0.001 per cent C_{16-18} fatty acids, 0.35 per cent glucose 1–1.2 per cent agar 5 per cent defibrinated horse blood) were used. As this substrate does not permit growth of *H influenzae* due to the lack of NAD 100 μl of a NAD solution (Merck) was spread on the agar surface before the inoculum containing approximately 10^7 cells/ml was added. After incubation for 24 hours at 37 C in an atmosphere containing 10 per cent CO_2, the inhibition zones were measured.

β-lactamase was determined by adding a drop of the chromogenic cephalosporin 87/312 Glaxo (9) to 24 hour colonies grown on Levinthal agar A red colour indicated the presence of a β-lactamase.

RESULTS

With one exception, all of the 148 strains of *H influenzae* could be assigned to one of *Kilian*'s five biotypes (I V) (5) on the basis of their biochemical reactions (Table 1) The correlation between the biotypes and the sites from which they were isolated is shown in Table 2. Fifty two strains belonged to biotype I Among these were 15 out of the 16 meningitis strains and the two strains from blood cultures the greater part of the remaining strains came from the respiratory tract. Biotype II consisted of 54 strains, 42 of which were isolated from the respiratory tract and seven from patients with otitis The 33 strains of biotype III were with few exceptions, isolated from the respiratory tract Biotype IV comprised four strains, one isolated from a case of meningitis and the other three from the respiratory tract. All of the four biotype V strains came from the respiratory tract. The sole remaining strain that could

TABLE 1. *Characteristics of H. influenzae Biotypes*

	Number of strains	Haemin requirement	NAD requirement	Haemolysis	Indole	Urease	Ornithine decarboxylase	Glucose	Sucrose	Lactose	Xylose	Mannitol	Serotype b capsule
Biotype I	52	+	+	—	+	+	+	+	—	—	+	—	22
Biotype II	34	+	+	—	+	+	—	+	—	—	d+	—	
Biotype III	33	+	+	—	—	+	—	+	—	—	d+	—	
Biotype IV	4	+	+	—	—	+	+	+	—	—	+	—	1
Biotype V	4	+	+	—	+	—	+	+	—	—	+	—	
Unclassified strains	1	+	+	—	—	—	+	+	—	—	+	—	

+ = 95–100 per cent positive d+ = 75–95 per cent positive, d = 25–75 per cent positive, d— = 5–25 per cent positive, — = 0–5 per cent positive.

not be fitted into any of the five biotypes was indole- and urease negative, ornithine decarboxylase positive and fermented glucose and xylose but not lactose, sucrose or mannitol.

Twenty-two out of 52 biotype I strains possessed a type b capsule 18 of these strains came from cases of meningitis and bacteraemia, while the last four were isolated from the respiratory tract. Only one other strain in the material was found to possess a type b capsule—a biotype IV strain isolated from a case of meningitis.

None of the 148 Danish strains gave inhibition zones smaller than 30 mm and, consequently the strains were all considered ampicillin sensitive. None of these produced β lactamase. Neither of the two ampicillin resistant reference strains gave visible inhibition zones and both were shown to produce β lactamase.

DISCUSSION

In complete accordance with *Kilian* (5) we found that, out of the theoretically possible eight biotypes based on the indole, urease and ornithine decarboxylase tests, three (I, II and III) accommodated most of the strains, a further two (IV and V) accommodating the remaining strains except one. This single strain has properties corresponding to three isolates of *H influenzae* which *Kilian* tentatively described as a sixth biotype.

Like the strains examined by *Kilian* all our biotype I II and V strains produced acid

TABLE 2. *Correlation between Origin of Strains and H. influenzae Biotypes*

Source of strains	Number of strains	*Haemophilus influenzae* biotypes I	II	III	IV	V	Unclassified strains
Meningitis	16	15 (94 %)	–	–	1 (6 %)	–	
Respiratory tract	114	33 (29 %)	42 (37 %)	31 (27 %)	3 (2.5 %)	4 (3.5 %)	1 (1 %)
Sputum	86						
Other	28						
Otitis media	8	1 (12 %)	7 (88 %)	–			
Blood cultures	2	2	–	–			
Other infections	8	1	5	2		–	
Total	148	52	34	33	4	4	1

from xylose in 5 days, while a few strains of biotypes II and III did not. However incubation of the apparently xylose negative cultures for an additional 10 days caused acid —in visually detectable amounts—to be produced by all strains. It may therefore be concluded *that all H influenzae* strains can produce acid from xylose, although the amounts produced in some cases are too small to be detected by the standard procedure.

With regard to the correlation between the biotypes and the origin of the strains, a comparison between our results and those obtained by *Kilian* is possible only in the case of strains from meningitis and from the respiratory tract because the number of strains from sources other than these is very small in our material. If only the recent isolates of *Kilian* from 1970–1972, are taken into consideration, it will be seen that 26 of the strains were derived from cases of *meningitis*, and 23 (88 *per cent*) *of these* were of biotype I We found that 15 out of 16 meningitis strains (94 per cent) belonged to biotype I. This indicates a definite dominance of biotype I from cases of meningitis. As to strains from the respiratory tract, 12, or 23 per cent, of *Kilian's* recently isolated 52 strains are assigned to biotype I 29 or 56 per cent, to biotype II, 7 or 13 per cent, to biotype III and 4 or 8 per cent, to biotype IV This does not differ much from our comparable values according to which 29 per cent belong to biotype I, 37 per cent to biotype II 27 per cent to biotype III and 2.5 per cent to biotype IV In addition our material also includes 4 strains (3.5 per cent) belonging to biotype V *Kilian's* hypothesis according to which there is some degree of correlation between a particular biotype of *H influenzae* and its source of origin is thus confirmed as far as our results are concerned.

The production of β lactamase has been shown to be R factor mediated and to be transferred widely among Gram negative bacteria (11) Thus, although we have not found any ampicillin resistant strains in our recently isolated material, the possibility that they may occur in the near future cannot be ignored.

ADDENDUM

Since submitting this manuscript we have received three ampicillin resistant *H influenzae* strains isolated in Denmark, one a type b strain belonging to biotype I and two non-type b strains belonging to biotype II

We sincerely thank the Heads of Departments of all the Regional Laboratories of the State Serum Institute who supplied most of the strains.

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THE FINE STRUCTURE OF CELLS OF *TRICHOMONAS VAGINALIS DONNÉ* OBTAINED FROM THE EXPONENTIAL PHASE OF GROWTH AND FROM STATIONARY CULTURES

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Nielsen, M. H. The fine structure of cells of *Trichomonas vaginalis Donné* obtained from the exponential phase of growth and from stationary cultures. Acta path. microbiol. scand. Sect. B, 84 205–216, 1976.

In cells of *Trichomonas vaginalis* in logarithmic phase of growth (log. *T. vaginalis*) the number of free cytoplasmic ribosomes was higher than that in cells from stationary cultures (stat. *T. vaginalis*). Polyribosomes closely surrounding the chromatic granules were present in equal amounts in both categories of cells, which indicates that they have specific functions and are not directly involved in the protein synthesis of the growing cell. The volume of the Golgi region and of the food vacuoles were approximately twice as large in log. *T. vaginalis* cells as in stat. *T. vaginalis* cells. The round and slightly ovoid chromatic granules were smaller in log. *T. vaginalis* cells than in stat. *T. vaginalis* cells, whereas elongated granules were more numerous in the former than in the latter cell type. The elongated chromatic granules presented a coarser texture than ordinary round granules. The chromatic granules probably replicate by binary fission, *a process which seems to follow an internal reorganization of the granules*. Polysaccharides were demonstrated on the cell surface and on the membranes of the Golgi and endocytotic vesicles if sectioned cells were stained with phosphotungstic acid after inert dehydration and embedding in a water-soluble methacrylate. Ruthenium red and colloidal iron only showed affinity for the filamentous cell coat.

Key words: *Trichomonas vaginalis*; ultrastructure; hydrogenosome.

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Previous papers have dealt with the fine structure of *Trichomonas vaginalis* obtained from *in vitro* cultures (22, 24), growth *in vivo* (25) or after transfer from growth *in vivo* to growth *in vitro* (26).

When *T. vaginalis* obtained from the human vagina were transferred to growth *in vitro* the most obvious changes, among others, were that their free cytoplasmic ribosomes became more numerous, and that the *number of their cytoplasmic glycogen gran-*

ules was reduced. These changes probably indicate that the growth rate of *T. vaginalis* increases when they are transferred from vaginal secretion to Diamond medium (26). These findings also corroborate observations by *Honigberg* (14) who noted that newly isolated parasitic protozoa generally acquire a faster growth rate if they are maintained in axenic cultures for some time.

In order to investigate the correlation, if any, between growth rate and fine structure of *T. vaginalis* exponentially growing cells and cells obtained from stationary cultures were studied by means of electron microscopy.

Some of the results obtained, in particular those concerning the relation between growth rate and fine structure of the chromatic granules, have been described in detail previously (29).

However, the results of comparative studies of endocytotic capacity and surface coats of cells obtained from the two types of culture will be given in the present report.

MATERIALS AND METHODS

T. vaginalis strain no. 1711 and a pool of freshly isolated *T. vaginalis* from several individual isolations were obtained from the Neisseria Department, Statens Seruminstitut. Examination of *T. vaginalis* strain no. 1711 in the logarithmic phase of growth (log. *T. vaginalis*) and from stationary cultures (stat. *T. vaginalis*) was performed as follows: 1) cells from the exponential phase were harvested when the generation time was 3½ hours and the cell density approximately 8 × 10 cells per ml, 2) cells from the early stationary phase were harvested when the generation time was 24 hours and the cell density approximately 2 × 10⁶ cells per ml.

Duplicate cell counts were made on 1 ml samples using a Coulter Counter model F (28). The cells were grown in Diamond medium without agar in an atmosphere of 95 per cent N and 5 per cent CO as previously described (28).

Cytochemical staining was performed both on cells of strain 1711 and on cells of the previously mentioned pooled material. These cells were grown for 20–24 hours in 10 ml Diamond medium without agar in screw cap stoppered test tubes (30).

Preparation for Electron Microscopy

A) *Ordinary fixation*. The study of the fine structure was carried out on cells of strain 1711 pre-fixed in a 1:1 dilution with distilled water of Karnovsky fixative (15) or in a 1.5 per cent solution of glutaraldehyde in 0.1 M cacodylate buffer pH 7.2.

Unless otherwise stated, the cells were post-fixed in 1 per cent osmium tetroxide and embedded in Vestopal-W. Thin sections were stained with uranyl and lead salts before examination in the electron microscope.

B) *Alcian Blue*. Alcian blue staining of the cell coat material (34) was performed on cells of strain 1711 and on cells from the pool of freshly isolated strains. Cells were obtained by centrifugation (900 *G*) for 5 minutes and then fixed for 60 minutes at 4 C after resuspension in 1.5 or 3 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 6.1 with alcian blue in a final concentration of 1 or 2 per cent. After a brief wash in buffer, cells were postfixed and embedded as described under A).

C) *Colloidal iron*. Staining of cell coat material with colloidal iron (21) was performed on pooled *T. vaginalis* from different isolates. Cells were obtained by centrifugation (900 *G*) for 5 minutes and then suspended at approximately 37 C in 1.5 per cent Noble agar in Diamond medium. After solidification, agar blocks with living *T. vaginalis* were incubated in Diamond medium at 37 C for 1 to 18 hours, and then fixed in 3 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 6.5 for 60 minutes. After washing for 30 minutes in buffer pH 6.5 the blocks were left for 90 minutes in a freshly prepared colloidal iron solution (21) and were then briefly washed in 12 per cent acetic acid. Post-fixation and embedding was performed as described under A).

D) *Ruthenium red*. Staining of cell coat material with ruthenium red (18) was performed on pooled *T. vaginalis* from different isolates. Cells were obtained by centrifugation (900 *G*) for 5 minutes and then fixed for 45 minutes in 0.1 M cacodylate buffer pH 7.2, containing 1 per cent glutaraldehyde and 0.1 per cent ruthenium red. After centrifugation and embedding in Noble agar (1.5 per cent in cacodylate buffer) agar blocks with cells were post fixed for 3 hours in Michaelis buffer pH 7.2, containing 1.4 per cent osmium tetroxide and 0.1 per cent ruthenium red. The blocks were briefly washed in buffer and transferred to 70 per cent alcohol containing 0.1 per cent ruthenium red before they finally were dehydrated and embedded as described under A).

E) *Phosphotungstic acid*. Inert dehydration and staining with phosphotungstic acid (16, 33) was performed on pooled *T. vaginalis* from different isolates. Cells were obtained by centrifuga-

tion (900 G) for 5 minutes and pre-fixed for 60 minutes at 4 C in 2 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. They were then embedded in Noble agar and agar blocks were passed through a series of dilutions of methacrylic acid-2-hydroxyethylester (Fluka) in distilled water. The ratios of ester to water used were 1:2, 2:1, 9:1 respectively. The blocks were kept for 15 minutes in each concentration and for 60 minutes in 100 per cent methacrylic acid-2 hydroxyethyl ester. After dehydration, blocks were impregnated for 60 minutes in a 3:7 mixture of methacrylic acid-2-hydroxyethyl ester and methacrylic acid butyl ester (Fluka) and for 60 minutes in the same mixture containing 2 per cent Luperco (Fluka). The latter mixture was also used for embedding. Polymerization was carried out at 60° C overnight.

F) *Horseradish peroxidase.* Uptake of horseradish peroxidase *in vitro* (5) was studied on a pool of freshly isolated *T. vaginalis* strains. Cells were obtained by centrifugation (900 G) for 5 minutes. The pellet was resuspended in Diamond medium to which 15 mg per ml of horseradish peroxidase (Sigma grade 1) was added. After incubation for 30 minutes at 37 C the cells were centrifuged and fixed for 50 minutes at 4 C in 2 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. They were washed in buffer for 30 minutes and incubated for 20 minutes at 37 C in 0.05 M Tris/HCl buffer pH 7.2, which contained 0.05 per cent 3,3'-diaminobenzidine tetrahydrochloride and 0.01 per cent H_2O_2. Finally the cells were washed by centrifugation in cacodylate buffer post-fixed in osmium tetroxide and embedded in Vestopal-W as described under A).

Ultrathin sections of cells treated according to preparation methods B) C) and F) were post stained with uranyl and lead salts before examination in the electron microscope. Sections of cells treated according to method D) were examined unstained, whereas sections of cells treated according to method E) were examined after staining for 30–60 minutes with 5 per cent phosphotungstic acid in distilled water. Electron microscopy was performed by a Siemens Elmiskop 1A.

RESULTS

The size and the fine structure of the *nucleus* of log. *T. vaginalis* and of stat. *T. vaginalis* were identical. A single large nucleolus was visible in some interphase nuclei, in others, nucleoli were indistinguishable from small electron-dense clumps of chromatin present in the nucleoplasm.

Dividing cells were observed in logarithmic cultures only but informative sections of clear-cut division stages were rarely found. Distinct mitotic figures were never observed in the nucleus of dividing cells and condensed chromosomes were not present until at a late stage of the division (CR, Fig 1). Attachment structures which supposedly insert the chromosomes into the nuclear envelope could not be distinguished from the normal nuclear pores of interphase cells. The nuclear envelope of dividing cells was intact (NE, Fig. 1).

At a time when the cells presented a normal configuration of their axostyle/pelta complex they were found to possess a four plus one arrangement of the kinetosomes. Cells in a later stage of division were recognized either because they had only 3 kinetosomes located in a group at the end of a bundle of microtubules (BU Fig 1) or because a bundle of cross-sectioned microtubules was visible in a groove formed by the nuclear envelope.

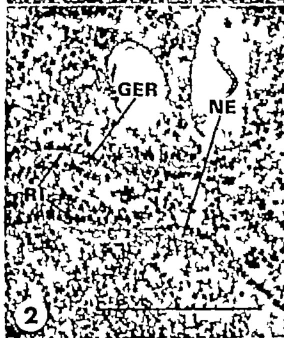
A mass of fine granular electron-dense material was located at the base of each group of kinetosomes (DM Fig. 1) and a bundle of microtubules together with free cytoplasmic microtubules (MT Fig. 1) were seen to radiate out from this dense material. At this stage of division, the microtubules of the axostyle and/or the pelta were not present around the kinetosomes.

The extensions of the *Golgi region* of log *T. vaginalis* averaged about 3.9 × 1.5 × 0.7 µm and of stat. *T. vaginalis* 2.7 × 1.2 × 0.5 µm. Calculated from these values, the "volume" of the Golgi region of log. *T. vaginalis* was about twice the "volume" of the Golgi region of stat. *T. vaginalis.*

Apart from this difference in size the ultrastructure of the Golgi regions of the two cell groups was identical.

The amount of *granular endoplasmic reticulum* (GER, Figs. 2 4 5) was nearly identical in cells from logarithmic and stationary cultures. The polyribosomes which were attached to the membranes of the endoplasmic reticulum were present as spirals or slightly curved structures (RI Fig 3).

The *free cytoplasmic ribosomes* were more


GER
NE
RI
2


NE
RI
3

numerous in log. *T vaginalis* (RI Fig 4) than in stat. *T vaginalis* (RI, Fig 5) but the number of polysomes closely surrounding the chromatic granules was about the same in both types of cells.

The cell membrane of both log. *T vaginalis* and stat. *T vaginalis* was covered, in patches, by a filamentous cell coat (4 23) Only this cell coat showed an obvious affinity to alcian blue, ruthenium red and colloidal iron, whereas the uncoated plasma membrane did not show any affinity (Figs. 8, 9) After inert dehydration and embedding in water miscible methacrylate, the exterior part of the cell membrane, and the luminal side of the membrane of endocytotic vesicles (FV) stained intensely with phosphotungstic acid (Fig. 10) Also the contents of the most peripherally located Golgi sacculess and vesicles were stained with phosphotungstic acid (Fig 10)

Figs. 1 9 and Figs. 11 16 are electron micrographs of ultrathin sections of *T vaginalis* cells embedded in Vestopal-W Fig 10 shows a part of section of a cell embedded in a water-miscible methacrylate. Unless otherwise stated, sections were post-stained with uranyl acetate and lead salts. The bar on each micrograph represents 0.5 μm.

Fig 1 An area of *T vaginalis* cell from an exponentially growing culture. The cell is in an early stage of division. A bundle of parallel microtubules (BU) and few single microtubules (MT) radiate from the electron-dense material (DM) located at the base of the kinetosome (K) The bundle of microtubules runs along the nuclear envelope (NE) The electron-dense fibre is probably a kinetosomal rootlet fibre but may represent a small tractophore (AT) The microtubules of the pelta and/or the axostyle are not visible near the kinetosomes. Small aggregates of chromatin (CR) are seen in the nucleus. Magn. 31,000 ×

Figs 2 and 3 Areas of *T vaginalis* cells from exponentially growing cultures. In Fig. 2, the perinuclear granular endoplasmic reticulum (GER) and the nuclear envelope (NE) are cross sectioned. The polyribosomes (RI) are directly attached to the outer surface of the membranes. In Fig 3, the nuclear envelope (NE) tangentially sectioned and the polyribosomes (RI) are visible as irregular spirals. Magn. Fig 2 41,000 × Magn. Fig 3: 48,000 ×

The *food vacuoles* of log *T vaginalis* were consistently larger than the food vacuoles of stat. *T vaginalis*. In the former group of cells, they occupied 7 1 per cent, in the latter 2.9 per cent of the cytoplasmic volume.

When horseradish peroxidase was added to living log *T vaginalis*, peroxidase reaction product was demonstrated on the surface of the cell membrane (arrow Figs. 11 & 12) and in particular within the patches of the filamentous cell coat material (CC, Fig 12 & compare with Fig 13) After 30 minutes of incubation, peroxidase reaction product could also be demonstrated in the interior of large food vacuoles (FV Fig 11) When added to stat. *T vaginalis* cultures, horseradish peroxidase was absorbed to the cell surface only and did not appear in the large food vacuoles within 30 minutes of incubation.

The *chromatic granules*—hydrogenosomes (17) —were smaller in log *T vaginalis* than in stat. *T vaginalis* In general, these granules were spherical or slightly ovoid in shape but in log *T vaginalis* about 3 per cent and in stat. *T vaginalis* about 0.5 per cent were more elongated (arrow Fig 14 CG Fig 16) or sometimes even dumb-bell shaped. The contents of the elongated granules were less electron-dense than the contents of the majority of the spherical granules and the texture of these granules was coarser A few spherical chromatic granules of log *T vaginalis* also displayed a coarse texture (Fig 15) but this type of chromatic granule was not found in stat. *T vaginalis* The chromatic granules in both groups of cells were surrounded by a limiting unit membrane 6 to 7 μm wide (L, Figs. 6 7) In certain regions this membrane was covered by some osmiophilic granules (OG Figs. 6 & 7)

DISCUSSION

The division rate of *T vaginalis* cells grown *in vitro* in logarithmic cultures decreases with the age of the culture. The reason for this is either that the available nutrients become sparse or that harmful metabolic end-products


CM
GV
FV
GS
8
9
10
FV
CC
11
12
13



Fig 14 Area of a *T vaginalis* cell from an exponentially growing culture. Note the difference in texture of the elongated chromatic granule (arrow) and the spherical or slightly ovoid paraxostylar chromatic granules (CG) The latter have a denser appearance. The nucleus (N) and the axostyle (AX) are also visible Magn. 30,000 ×

Fig 15. Area of *T vaginalis* cell from an exponentially growing culture Note the difference in texture of the chromatic granules (CG) Magn. 62,000 ×

Figs. 8 and 9 Areas of *T vaginalis* cells from exponentially growing cultures The cell in Fig 8 was prefixed in buffered 2 per cent glutaraldehyde which contained 2 per cent alcian blue. The cell in Fig 9 was pre fixed in 2 per cent glutaraldehyde only The appearance of the cell membranes and of the cell surfaces is identical in the two cells. No cell coat material is visible. Magn. Figs. 8 and 9 43,000 ×

Fig 16. Area of a *T vaginalis* cell from an exponentially growing culture which was fixed in glutaraldehyde embedded in water-miscible methacrylate after inert dehydration and post-stained with phosphotungstic acid. The cell membrane (CM) and the inside of the limiting membrane of the food-vacuoles (FV) are stained with the phosphotungstic acid. This also applies to the more peripherally located saccules (GS) and vesicles (GV) of the Golgi region. Magn 22,000 ×

Fig 11 Area of a *T vaginalis* cell from an exponentially growing culture The cells were incubated with horseradish peroxidase for 30 minutes before fixation. Peroxidase reaction product is confined to the cell surface (arrow) and to th food-vacuoles (FV) Magn. 40 000 ×

Figs. 12 and 13 Areas of *T vaginalis* cells from a stationary phase culture. The cell in Fig. 12 was incubated with horseradish peroxidase for 30 minutes before fixation. The cell in Fig. 13 was incubated in medium without peroxidase. In Fig. 12 peroxidase reaction product is confined t the surface of the plasma membrane (arrow) and to the filamentous cell coat (CC) In Fig. 13 no reaction product can be seen. Magn. Fig. 12 43,000 × Magn. Fig. 13: 40,000 ×


CM
GV
FV
GS
CC
FV
8
9
10
11
12
13



Fig. 14. Area of a *T. vaginalis* cell from an exponentially growing culture. Note the difference in texture of the elongated chromatic granule (arrow) and the spherical or slightly ovoid paraxostylar chromatic granules (CG). The latter have a denser appearance. The nucleus (N) and the axostyle (AX) are also visible. Magn. 30 000 ×

Fig. 15. Area of a *T. vaginalis* cell from an exponentially growing culture. Note the difference in texture of the chromatic granules (CG). Magn. 62,000 ×

Figs. 8 and 9. Areas of *T. vaginalis* cells from exponentially growing cultures. The cell in Fig. 8 was prefixed in buffered 2 per cent glutaraldehyde which contained 2 per cent alcian blue. The cell in Fig. 9 was pre-fixed in 2 per cent glutaraldehyde only. The appearance of the cell membranes and of the cell surfaces is identical in the two cells. No cell coat material is visible. Magn. Figs. 8 and 9 45,000 ×

Fig. 10. Area of a *T. vaginalis* cell from an exponentially growing culture which was fixed in glutaraldehyde, embedded in water-miscible methacrylate after inert dehydration and post-stained with phosphotungstic acid. The cell membrane (CM) and the inside of the limiting membrane of the food-vacuoles (FV) are stained with the phosphotungstic acid. This also applies to the more peripherally located saccules (S) and [illegible] vesicles (GV) of the Golgi region. M[illegible] 000

Fig. 11. Area of a *T. vaginalis* cell from an exponentially growing culture. The cells were incubated with horseradish peroxidase for 30 minutes before fixation. Peroxidase reaction product is confined to the cell surface (arrow) and to the food-vacuoles (FV). Magn. 40 000 ×

Figs. 12 and 13. Area of *T. vaginalis* cells from a stationary phase culture. The cell in Fig. 12 was incubated with horseradish peroxidase for 30 minutes before fixation. The cell in Fig. 13 was incubated in medium without peroxidase. In Fig. 12 peroxidase reaction product is confined to the surface of the plasma membrane (arrow) and to the filamentous cell coat (CC). In Fig. 13 no reaction product can be seen. Magn. Fig. 12 45,000 × Magn. Fig. 13 40,000



Fig. 16. Area of *T. vaginalis* cell from an exponentially growing culture. The elongated chromatic granule (CG) presents a rather loose texture. Magn. 50,000 ×

granules. The morphological events of this replication seem to be a progressive elongation of the granules which is later followed by fission at the mid region (29). The chromatic-granule-like "mitochondria" which are located in cells of the hypermastigid protozoan *Joenia*, apparently divide in this way (10). In the present study a variation in the texture of individual chromatic granules was noted. Furthermore, the appearance of a granule was correlated with the state of replication of this granule. Such a correlation has not been demonstrated previously. It is tempting to compare such an internal reorganization of the chromatic granules to the morphological changes which were seen to occur in dividing mitochondria of Boderia (8).

Cells from different *T. vaginalis* isolates were examined previously (22). In retrospect a few of these seem to be outstanding because in their cells the chromatic granules were irregularly shaped and all cells also contained a high number of elongated granules. The contents of these granules, however, did not display the morphological variations presently observed in cells obtained from exponentially growing cultures.

Information on the division stages of the trichomonads are difficult to obtain by electron microscopy of sectioned cells. From the present and previous studies (17, 13, 19, 22, 21) it is evident, however, that the first sign of division in *Trichomonadinae* cells is a duplication of the parabasal filament and possibly also the costa. Later, a sixth kinetosome is formed. This is first demonstrated at a time when the five interphase kinetosomes still had a normal spatial relationship mutually as well as with respect to the axostyle/pelta complex (27). Still later, two groups with three kinetosomes each are formed and, at the same time, the axostyle/pelta complex has disappeared from the cytoplasm surrounding the kinetosomes. At this stage of division there is no evidence of duplication of the axostyle/pelta complex in *T. vaginalis* cells otherwise seen in the case of monoceromonas (20).

Some unpublished results and the present investigation confirm that cells of *T. vaginalis*, like those of other members of the order Trichomonadida and Hypermastigida, have extranuclear division "spindles" and that their nuclear membranes remain intact during a complete cell division cycle. The compact bundle of spindle microtubules demonstrated in the present study probably correspond to the "desmosomal" or "centrodesmosomal" fibre previously described in light microscopy studies of dividing *T. vaginalis* cells (3, 7). The location of the kinetosomes together with the electron-dense material, from which the bundle of microtubules in dividing *T. vaginalis* cells "originates" resembles that seen in the pericentriolar electron-dense material of normal mitotic cells (6). Rather one would expect a more close

similarity to the previously described arrangement of the atractophores of some Hypermastigida (9 11) but such an arrangement could not be demonstrated in cells of the present material. Similarly any special attachment structures between centromeres and spindle microtubules as seen in the nuclear envelope of the cells of some Hypermastigida (11) were not observed in dividing *T vaginalis* cells.

The author is indebted to Dr *Roderic Pontefract* Microbiology Research Division, Health and Welfare Ottawa, Canada, and to Mr *Aksel Birch Andersen* Biophysical Department, Statens Seruminstitut, Copenhagen, for valuable criticism during the preparation of the manuscript.

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Fig. 16. Area of *T. vaginalis* cell from an exponentially growing culture. The elongated chromatic granule (CG) presents a rather loose texture. Magn. 50,000 ×

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LOCALIZATION OF UREASE ACTIVITY IN *UREAPLASMA UREALYTICUM* CELLS

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Vinther, O. Localization of urease activity in *Ureaplasma urealyticum* cells. Acta path. microbiol. scand. Sect. B, 84: 217-224, 1976.

Measurements of urease activity of various cell fractions of *U. urealyticum* showed that this activity was confined to the soluble fraction of the cytoplasm. It was attempted to devise a method for electron microscopic detection of the sites of urease activity based on precipitation of electron dense MnO_2 at the alkaline pH created by hydrolysis of urea. The results obtained by this method supported the previous results indicating a cytoplasmatic localization of the urease activity in the cells. Helical ribosome patterns were observed when glutaraldehyde fixed cells were treated with the cytochemical test solutions.

Key words: *Ureaplasma urealyticum* cells; urease activity; localization.

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The human T-mycoplasmas have recently been assigned to a separate genus *Ureaplasma* of the family *Mycoplasmataceae* (24) primarily on the basis of their ability to hydrolyse urea (6, 22). Possession of urease activity distinguishes the T-mycoplasmas from all other members of the order *Mycoplasmatales*. The genus *Ureaplasma* contains one species, *Ureaplasma urealyticum* (24) comprising eight serotypes (2).

It has not been definitely established whether urea is an absolute requirement of *U. urealyticum* for growth and multiplication (16, 17, 18) or urea just has an enhancing effect on the growth of these organisms (6, 22). Radioactive carbon is not incorporated in the cells during growth in ^{14}C-urea containing media (7), and nearly all nitrogen from hydrolysed urea is apparently released as free NH_4 (6, 7). The possibility exists that urea may serve as an energy source for *U. urealyticum* since about 15 kCal are released when 1 mole of urea is hydrolysed to CO_2 and NH_3 at pH 6 (11); however, no chemical pathway is known by which this energy can be utilized. Attempts to detect enzymes which might link the breakdown of urea through carbamate to the formation of energy-rich carbamoylphosphate (carbamate kinases, EC 2.7.2.2.) using radioactive labelled substrates, have failed (*Vinther*, unpublished experiments).

To obtain some further information about the urease activity of *U. urealyticum* it was decided to investigate the localization of this activity within the cells.

Since it was desirable to sup- the direct chemical measurements of tions with a method not invo

tionation it was attempted to adapt the method introduced by *Shepard & Howard* (23) for light microscopic identification of *U urealyticum* colonies to electron microscopy. This method is based on precipitation of MnO_2 which is electron opaque at the alkaline pH created by hydrolysis of urea.

MATERIALS AND METHODS

Organisms. Strain Pirillo (serotype VI) and Cook (serotype VII) of *U urealyticum* (2) were used in the investigation. *M mycoides* subsp. *capri* (PG3) was included in the cytochemical study as a representative of mycoplasmas not showing urease activity. The *U urealyticum* strains were cultivated for electron microscopic purposes on solid S medium (26). Plates were inoculated with 0.05 ml of a 1:500 dilution of stock cultures containing approximately 5 × 10⁸ CFU/ml and incubated for 7 days at 32 C in an atmosphere of 94.4 per cent N_2 + 5.6 per cent CO_2. The low temperature and anaerobic atmosphere favoured the development of colonies larger than those observed under normal conditions. Well separated colonies with diameters ranging from 150 µm to 250 µm were selected for electron microscopic examination. For measurements of urease activity following cell fractionation strain Cook was grown for 12 hours at 37 C in 5 l of liquid S medium. *M mycoides* subsp. *capri* was cultivated on solid B medium (5).

Cell fractionation. To separate different fractions of *U urealyticum* cells, 5 l of a culture in the logarithmic growth phase were harvested by continuous-flow centrifugation at 48200 *g* in a Sorvall RC2B centrifuge. The pellet was washed once in 30 ml 0.15 M NaCl and resuspended in 6.0 ml 0.15 M NaCl. 2.0 ml of this suspension was pelleted at 27000 *g* and the pellet resuspended in 2.0 ml redistilled water. These processes were all carried out at +4 C. To bring about cell lysis, the suspension in water was incubated for 30 min at 37 C and immediately afterward subjected to 20 times alternate freezing at −70° C and thawing at room temperature. Membranes were collected by centrifugation at 37000 *g* for 40 min and resuspended in the original volume of distilled water (2.0 ml). Urease activity of the membrane fraction was measured both before and after washing with 0.15 M NaCl. The activity of the corresponding supernatant (cytoplasm) fraction was likewise measured. A ribosome fraction was prepared from this cytoplasmic fraction by centrifugation for 2 hours at 100000 *g* in a Sorvall OTD-2 ultracentrifuge and resuspension in the original volume of distilled water. The various fractions were examined by electron microscopy after staining with ammonium molybdate (1) and viable counts were performed on samples of each fraction.

Measurement of urease activity. The test solution by which to measure urease activity of whole cell suspensions and cell fractions consisted of 0.500 ml 0.05 M phosphate buffer containing 0.05 M unlabelled urea and 0.2 µCi ¹⁴C labelled urea (2.94 µCi/µmole). The pH of the mixture was 6.1. To start the reaction, 10 µl cell suspension or cell fraction was added to the test solution and the test tube was closed with a rubber stopper through which the needle of a syringe containing 0.1 M $Ba(OH)_2$ was inserted. The tube was incubated for 20 min at 37 C and the reaction was stopped by injecting 0.5 ml $Ba(OH)_2$ from a syringe into the assay solution. The precipitate of radioactive $BaCO_3$ formed was retained by filtration through a small 100 nm Millipore filter and washed twice with $Ba(OH)_2$. The filter was transferred to a counting vial and counted in 10 ml INSTA-GEL (Packard) in a Packard Tri-Carb liquid scintillation spectrometer. The filtrate and combined washing solutions were likewise counted. The results were corrected for quenching by the channel ratio method.

The amount of protein in the assay mixture was adjusted so that the decomposition of urea was less than 25 per cent. In this range activity was found to be proportional to the protein concentration. Cell protein suspensions heat treated at 60 C for 30 min were used as negative controls. The accuracy of the method was determined using ¹⁴C labelled $NaHCO_3$. Less than 0.2 per cent activity was found in the filtrate after precipitation with $Ba(OH)_2$. Between 1.0 per cent and 1.5 per cent of urea ¹⁴C activity was constantly bound to filters in experiments where no protein or heat denaturated protein was added. Correction was made for this unspecific binding. Although the partial pressure of CO_2 in the assay mixture is not negligible any escape of ¹⁴CO_2 activity could not be detected due to the closed system used. If the rubber stopper was removed prior to the addition of $BA(OH)_2$, significant amounts of radioactivity would be lost.

To measure the effect of glutaraldehyde treatment on the urease activity of cells, glutaraldehyde (25 per cent) was added to whole cell suspensions to make the final concentrations of glutaraldehyde in the suspensions 0.3 per cent and 3 per cent, respectively. Cells were incubated in the fixatives for one hour at room temperature immediately prior to urease activity assays. The effect of substituting the veronal-acetate buffer used in the electron microscopic study for phosphate buffer in the assay mixture was likewise determined.

Preparation of specimens for electron microscopy. Colonies to be examined by electron microscopy were prefixed *in situ* by gently applying a 0.3 per cent solution of glutaraldehyde in veronal-acetate buffer

containing 0.01 M $CaCl_2$, pH 6.1 (VA buffer) (21) onto the agar surface. Prefixation proceeded for one hour at room temperature after which time the excess of glutaraldehyde was removed and the plates were dried at 37 C for 30 min. Colonies selected for examination were then covered with 1 or 2 drops of warm (45 C) 1 per cent Noble agar (Difco) in VA buffer (4) and, after solidification, small agar blocks with a single colony embedded were cut out and fixed for one hour at room temperature in 3 per cent glutaraldehyde in VA buffer. Further fixation in 1 per cent OsO_4 solution containing yeast extract—sodium acetate—peptone medium (10) and treatment with 2 per cent uranyl acetate (21) took place as described elsewhere (4). Dehydration was carried out in a graded series of acetone—H_2O mixtures and, finally blocks were embedded in Vestopal—W. Sections were obtained with an LKB Ultrotome III microtome and examined either unstained or after post-staining with magnesium uranyl acetate (8) and lead citrate (20) in JEOL JEM 100B electron microscope.

Cytochemical localization of urease activity. Electron microscopic localization of urease activity in *U urealyticum* was assessed after incubation of colonies with solutions containing Mn^{2+} ions and urea (23). To investigate the effect of glutaraldehyde fixation on localization and enzymatic activity *Ureaplasma* colonies were incubated with Mn^{2+}-urea solutions either before prefixation with 0.3 per cent glutaraldehyde as described or immediately after prefixation and drying. The test solutions were applied onto the agar surface by a pasteur pipette. In some experiments, small agar blocks containing a single colony fixed in 3 per cent glutaraldehyde were incubated with Mn^{2+} and urea.

Colonies were incubated for either 10 min or 30 min at 37 C, and reactions were terminated by briefly washing the colonies in VA buffer. Two different Mn^{2+}-urea solutions were used. The first solutions was 1 mM with respect to Mn^{2+} and 10 mM with respect to urea and was mostly used in experiments with unfixed cells. Most incubations of glutaraldehyde fixed cells were carried out in solutions 5 mM and 20 mM with respect to Mn^{2+} and urea. Both solutions further contained $CaCl_2$ (0.01 M) and NaCl (0.15 M) to maintain proper osmotic conditions, pH was adjusted to 6.1. Control experiments were performed in which (i) both Mn^{2+} and urea were omitted, (ii) Mn^{2+} was omitted (iii) urea was omitted from the reaction mixture.

Chemicals. All chemicals used were of analytical grade. Unlabelled urea (Merck) and ^{14}C labelled urea (NEN Chemicals GmbH) were employed. The radiochemical purity of urea ^{14}C was stated to be greater than 98 per cent. The source of Mn^{2+} ions in cytochemical investigations was $MnCl_2 \cdot 4H_2O$ (Merck).

RESULTS

Measurements of urease activity following cell fractionation. Examination by way of electron microscopy of whole cell suspensions revealed the presence of numerous filamentous cells (1) and very little material which could be interpreted as flat or deteriorated membranes. After osmolysis and freeze-thawing, grids would be crowded with apparently flat membranes and fragments of membranes dark coloured on a light background. The ribosome fraction contained positively stained ribosome particles with diameters ranging from 14 nm to 17 nm (9) mostly arranged in clusters of 3 or more particles. No membranes or whole cells were seen in these preparations.

Viable organisms were not found in cell suspensions after osmolysis followed by freeze thawing or in any of the cell fractions.

The results of measurements of urease activity of the various fractions of *U urealyticum* cells are shown in Table 1. The residual activity found in the membrane fraction after one wash constituted 4 per cent of the activity of the suspension of broken cells. A considerable decline in activity of this suspension

TABLE 1 *Urease Activity of* U urealyticum *(Cook) Cell Fractions*

Fraction	Urease activity (% decomposition of urea in assay solution)
Suspension of whole cells	21.5 ± 2*
Same after osmolysis + freeze-thawing	7.5 ± 1
Cytoplasmic fraction	5.1 ± 1
Membrane fraction before wash	0.9 ± 0.5
Membrane fraction after wash	0.3 ± 0.5
Ribosome fraction	0‡

* The average urease activity per cell was 2.6 × 10^{-1} moles urea hydrolysed/min/cell.

‡ Activity indistinguishable from that of heat denatured protein control.


1


D
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D
H
2


C
3


A
5


4

relative to the activity of the suspension of whole cells was noted. No ribosome bound urease activity could be detected.

Cytochemical localization of urease activity After incubation in the Mn^{2+} urea test solutions the *Ureaplasma* colonies acquired a golden brown coloration (23) which after short incubation times would be most intense in the central ("yolk") part of colonies showing typical fried-egg appearance. On prolonged incubation, coloration increased in intensity and tended to become uniform, although still strictly confined to the colonies (23). Incubation with Mn^{2+} alone or urea alone produced no visible change in colony appearance.

Fig. 1 shows a micrograph of untreated *U urealyticum* cells surrounded by a triple-layered membrane and possessing on the outer side of the membrane an electron-dense layer about 13 nm thick (1). After incubation of unfixed cells with Mn^{2+}-urea solutions, a general increase in the electron scattering power of the cell cytoplasm was noted, most pronounced in the dense areas of the cytoplasm, (Fig 2). In some cells, mostly small cells, clusters of an apparently microcrystalline precipitate of highly electron opaque material were observed in the cytoplasm (Fig 3). Cell morphology was not completely preserved in preparations of *U urealyticum* cells incubated with the test solutions prior to fixation. In a considerable number of cells, leakage of intracellular material through holes in the membranes was evident (Fig 2) still, undamaged cells containing deposits of electron dense material were also observed, e.g the cell in Fig 3.

To a smaller extent, a general contrast enhancement of the cytoplasm was also visible in cells incubated with Mn^{2+} alone, whereas urea alone did not produced any change in cell appearance. No cells containing microcrystalline precipitates were found in any of the control preparations neither was any difference between unincubated cells of *M mycoides* and cells incubated in the test solutions observed.

Colonies treated with Mn^{2+} urea solutions after prefixation in 0.3 per cent glutaraldehyde also developed a golden brown colour although somewhat less intense than the colour acquired by unfixed colonies. Fixation in 3 per cent glutaraldehyde darkened the colonies and the surrounding agar and any effect of incubation with the test solution could not be observed in the light microscope.

The most prominent structural difference between untreated cells and cells treated with Mn^{2+} and urea either after fixation in 0.3 per cent glutaraldehyde or after fixation with 3 per cent glutaraldehyde was the occurrence of regular geometric patterns of ribosomes in the latter cells. These patterns are illustrated in Fig 4 showing a wave formed possibly helical arrangement of ribosomes. A detailed

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Fig 1–5 show thin sections of *U urealyticum* cells which has been treated with the cytochemical test solutions as indicated. Sections were poststained with magnesium uranyl acetate and lead citrate. The bar on each micrograph represents 0.1 µm.

Fig 1 Untreated cells of strain Cook, showing cytoplasmic membrane and an extramembranous electron dense layer about 13 nm thick. × 55000.

Fig 2 Strain Pirillo cells incubated for 10 min in 1 mM Mn^{2+} + 10 mM urea prior to any glutaraldehyde fixation. Electron dense deposits (D) assumed to be MnO are seen in the cytoplasm of the large as well as the neighbouring small cell. Holes (H) in the membrane of the large cell through which leakage of intracellular material has occurred are evident. × 70000

Fig 3. Same strain and same treatment as in Fig. 2 One cluster (C) of apparently microcrystalline material (MnO) together with tiny electron dense patches scattered rather uniformly throughout the cytoplasm is observed. × 130000.

Fig 4 Strain Cook incubated with 5 mM Mn^{2+} + 20 mM urea for 30 min after fixation in 3 per cent glutaraldehyde. Ribosomes arranged in a wave-formed pattern are seen near one end of the elongated cell. Precipitates of electron dense material were not observed in these preparations. × 65000

Fig 5 Strain Cook incubated with 5 mM Mn^{2+} + 20 mM urea for 10 min after prefixation in 0.3 per cent glutaraldehyde. The high contrast of the dense areas of the cytoplasm is assumed to be due to staining of these areas (A) by urease reaction products. × 70000.

analysis of this arrangement was not attempted. In cells where the clusters were properly oriented relative to the plane of sectioning individual ribosomes could be distinguished and were found to have diameters of approximately 15 nm. Ribosome patterns of this kind were found both in serotype VI and serotype VII of *U urealyticum* most frequently in small and medium sized cells with a uniformly dense cytoplasm. Ribosome helices were noticed to occur at about the same frequency in *U urealyticum* cells incubated with Mn^{2+} solution alone but were never observed in *M mycoides*

Some increase in contrast of the dense areas of the cytoplasm of cells prefixed in 0.3 per cent glutaraldehyde and subsequently incubated with Mn^{2+}-urea solutions was observed (Fig 5) However the gain in contrast was not quite as high as that seen in unfixed cells (Fig. 2) and no microcrystalline precipitates (Fig 3) were detected. Cellular morphology was well preserved and no contrast enhancement was apparent in any of the control preparations.

Increase in contrast could not be demonstrated in *U urealyticum* cells treated with the Mn^{2}-urea test solutions after fixation in 3 per cent glutaraldehyde.

The results of measurements of urease activity inhibition caused by glutaraldehyde fixation or by substituting VA buffer for phosphatase buffer in the assay mixture are presented in Table 2. Evidently fixation in 0.3 per cent glutaraldehyde for one hour caused a considerable loss in urease activity and no measurable activity was left after fixation in 3 per cent glutaraldehyde.

TABLE 2. *Inhibition of Urease Activity of* U urealyticum *(Cook) whole Cell Suspension by Glutaraldehyde and VA Buffer*

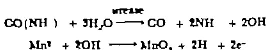
Cell treatment	Relative urease activity (%)
Untreated cells, assay in phosphate buffer	100
Untreated cells, assay in VA buffer	86
Cells fixed 1 h in 0.3 % glutaraldehyde, assay in phosphate buffer	32
Cells fixed 1 h in 3 % glutaraldehyde, assay in phosphate buffer	0

DISCUSSION

The results in Table 1 suggest that urease activity of *U urealyticum* is almost completely confined to the cytoplasm of the cells. The lower activity of the suspension of broken cells compared with the suspension of whole cells (Table 1) was at least in part due to thermal instability of the urease at room temperature since a decline in activity of all cell fractions after storage at room temperature was noted

Mycoplasmas are known to vary greatly in their ability to undergo osmotic lysis (19) The procedure used in this study to break up *U urealyticum* cells seemed to be efficient since (i) viable cells were not found in the suspensions of broken cells (3) (ii) no whole cells were seen in negatively stained preparations of these suspensions, (iii) urease activity of the membrane sediments was low

The sequence of chemical reactions which forms the basis of the cytochemical method of urease localization is



$$CO(NH\ ) + 3H_2O \xrightarrow{urease} CO\ + 2NH\ + 2OH$$
$$Mn^{2}\ + 2OH \longrightarrow MnO_2 + 2H\ + 2e^-$$

Insoluble manganese dioxide is formed as the end product of the reactions. Assuming an average cell volume of about 2 × 10⁻ cm^3 the number of Mn atoms bound to one cell calculated from the average urease activity per cell (Table 1) is estimated to be 10 to 10 times the minimal number required to obtain a just appreciable increase in contrast (25)

The attempts made in the present study to use these principles to obtain information about the ultrastructural localization of urease

activity in *U urealyticum* cells met with only partial success. The appearance of amorphous (Fig. 2) and microcrystalline (Fig 3) precipitates of electron dense material in the cytoplasm of cells treated with Mn^{2+} and urea prior to any fixation lends some support, however to the conclusion reached by measurements of urease activity i.e. that urease activity is found predominantly in the cytoplasm, although the unsatisfactory preservation of cell morphology observed in these preparations to a certain degree depreciates the reliability of the method. The contrast enhancement of the cytoplasm observed when cells prefixed in 0.3 per cent glutaraldehyde were incubated with the test solutions (Fig. 5) was generally less intense and of a more diffuse character which made interpretation difficult. A major reason for this relatively low increase in contrast is undoubtedly the substantial inhibition of urease activity caused by treatment of cells with 0.3 per cent glutaraldehyde (Table 2) This treatment may possibly also change the permeability of cell membranes for Mn^{2+} ions. The VA buffer used in the electron microscopic work did not significantly reduce urease activity (Table 2) The failure of cells incubated with the Mn^{2+} urea test solutions after fixation in 3 per cent glutaraldehyde to exhibit any increase in contrast is explained by the total inhibition of urease activity caused by this fixative (Table 2)

Since no contrast enhancement was observed in *M mycoides* cells incubated with Mn^{2+} and urea, the amount of unspecific staining was obviously minimal. The slight contrast increase seen after incubation of unfixed *U urealyticum* cells with Mn^{2+} alone may be ascribed to the effect of the urea contained in the medium (~ 1 mM) which could not be excluded. Incubation of cells with 10^{-} M mercuric chloride to inhibit urease activity (23) produced a contrast in the sections of a degree which prevented assessment of the effect of a *following* treatment with Mn^{2+}

The degree of localization of precipitated MnO_2 will depend among other things upon the rate of trapping of liberated OH by Mn^{2+} to form insoluble MnO_2 If the test solutions were applied to colonies, coloration of these would develop within less than one second.

Geometric ribosome arrangements have previously been observed in *M gallisepticum* (4 12, 13 15) and in 4 strains of *U urealyticum* (1) The helical nature of the arrangement in *M gallisepticum* was amply confirmed by diffraction measurements (14) whereas the arrangement in *U urealyticum* was in the form of tetrads and "corn-cob" like structures (1) In the present study a helical configuration was implied from micrographs of several cells in which the plane of sectioning was neither parallel nor perpendicular to the helices axes (Fig. 4) Any connection between the urease activity of *U urealyticum* cells and the formation of ribosome helices could not be demonstrated and no urease activity was detected in sedimented ribosomes (Table 1)

The skilful technical assistance of *Birthe Saby* in preparing specimens for electron microscopy is gratefully acknowledged.

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IMMUNOELECTROOSMOPHORESIS FOR DETECTION OF REO-LIKE VIRUS METHODOLOGY AND COMPARISON WITH ELECTRON MICROSCOPY

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Tufvesson, B. & Johnsson, T. Immunoelectroosmophoresis for detection of reo-like virus Methodology and comparison with electron microscopy Acta path. microbiol scand. Sect. B, 84 225–228, 1976.

In two separate studies, faecal samples were collected from children with acute gastroenteritis the samples were tested both by electron microscopy and immunoelectroosmophoresis with a view to detect reo-like virus. In 94 per cent and 100 per cent of samples positive by electron microscopy in the first and second material respectively a precipitin line was found after electrophoresis, using a guinea pig antiserum against a purified bovine virus. No false positives were detected by this method.

Key words: Reo-like virus gastroenteritis immunoelectroosmophoresis.

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In small children, gastroenteritis associated with the presence of reo-like virus in faeces is now a well-known illness, which has been reported from various parts of the world (6, 9) The diagnostic method used in most trials reported is electron microscopy (EM) of faecal samples. However this method requires expensive equipment and cannot be performed in all laboratories. A corresponding bovine virus, Nebraska Calf Diarrhoea Virus (NCDV) which can be cultured on calf kidney cells, was injected into guinea pigs by *Spence et al.* (8) and the resulting antiserum was used in complement fixation (CF) for direct detection of human virus. Culturing of human reo-like virus has until recently only been possible on organ cultures of human intestinal cells (10) By now however *Banatvala et al.* have presented a system for *in vitro* detection of human reo-like virus, which involves culturing on pig kidney cells plus an additional immunofluorescent test (1) The NCDV has also been used as an antigen, surveying paired sera by CF (5 9) and by immunofluorescence (11) The present work deals with immunoelectroosmophoresis (IEOP) a rapid and inexpensive alternative method for detection of human reo-like virus.

MATERIALS AND METHODS

Faeces: From a survey conducted November 1974–April 1975 114 faecal extracts from children with gastroenteritis were obtained, as well as 29 samples from children with other types of illness. The oc-

currence of infection caused by reo-like virus in this study carried out by EM and CF has been presented in a previous paper (9). In a second study from May 1975–January 1976 the number of faecal samples from patients with gastroenteritis totalled 130. In the first study the percentage of faeces in the extracts varied from a few per cent up to twenty per cent, due to shortage of material in the samples provided. In the second study more faecal material was provided in each separate case and it was always possible to make 20 per cent (w/v) solutions.

EM: EM of the faecal samples was carried out according to the method described by *Flewett et al.* (3). Half of the squares on each grid were examined at a magnification of 22,500 ×. The number of virus particles in a purified antigen, as well as in some faecal extracts, was counted by comparison with a Latex suspension (particles Ø 264 nm) of known concentration.

Antiserum: A bovine strain of reo-like virus (NCDV strain Lincoln) was cultivated in calf kidney cultures. After purification of the virus by treatment with Arklone and polyethylene glycol, as described by *Bishop et al.* (2), guinea pigs were given intramuscular injections on days 1, 15 and 29. The injections contained a mixture of Freund's complete adjuvant and 25 µg of the purified virus per animal. The animals were sacrificed on day 43. Double gel diffusion tests were performed using a micro-Ouchterlony technique described by *Prince* (7) in order to check the identity of antibody with a number of paired human sera from EM positive cases. Using faecal extracts as antigen, there was a sharp precipitin line against human convalescent sera, while there was no line between the antigen and the acute sera. Guinea pig antisera giving precipitin lines which showed identity with the convalescent sera were selected and pooled.

IEOP: The electrophoresis was performed according to a method described by *Hansen & Johansen* (4) in 0.75 per cent agarose (l'Industrie Biologique Francaise) in barbital buffer pH 8.6. Undiluted clarified faecal extracts were placed in circular wells and tested against an equal amount of undiluted guinea pig antiserum. Seventy samples could be tested on each plate. The electrophoresis was run for a period of 75 minutes at 7 V/cm. After electrophoresis, the plates were preliminarily read, submerged in saline overnight, stained with Coomassie brilliant blue and finally examined.

RESULTS

The results obtained in the first study are listed in Table 1. The number of positive samples after staining was 49 (81 per cent) of the 61 shown to be positive by EM. Eight of the remaining 12 which were positive by EM were also positive in IEOP after a tenfold concentration in Lyphogel. Four samples which were negative by EM but where corresponding paired sera showed significant rise in titre by CF were also negative by IEOP. In tests of 82 faecal samples containing other viruses, samples from patients negative with respect to gastroenteritis, or samples from patients with other diseases, no precipitin lines were found. The results obtained in the second study in which all the faecal extracts contained 20 per cent faeces are shown in Table 2. In this study all faecal samples were tested by IEOP directly on their arrival, and all specimens were checked secon-

TABLE 1. *Comparison of EM and IEOP for Detection of Reo-like Virus in 143 Faecal Samples (December 1974–May 1975)*

	EM result			
	Gastroenteritis			Other diagnoses
	Reo-like virus	Other viruses	No virus	
Total number tested	61	12	41	29
IEOP-positive before staining	6 (10%)	0	0	0
Additional IEOP positive after staining	43 (71%)	0	0	0
Additional IEOP-positive after concentration	8 (13%)	0	0	NT
Total IEOP-positive	57 (94%)	0	0	0

Corresponding paired sera from four patients showed significant rise in titre in CF.
NT = not tested

TABLE 2. *Comparison of EM and IEOP for Detection of Reo-like Virus in 130 Faecal Samples from Patients with Gastroenteritis (May 1975–January 1976)*

	EM result		
	Reo-like virus	Other viruses	No virus
Total number tested	63	6	61
IEOP-positive before staining	31 (49%)	0	0
Additional IEOP-positive after staining	32 (51%)	0	0
Total IEOP-positive	63 (100%)	0	0

darily by EM the IEOP result being unknown to the investigator In this study 31 samples (49 per cent) were found to be positive before staining and an additional 32 samples (51 per cent) were positive after staining, a total of 63 samples being tested, thus showing a 100 per cent correlation between IEOP and EM. No false positives were detected among 67 faecal samples from patients with other viruses or from patients negative with respect to gastroenteritis. Examination of precipitates from gel diffusion and IEOP experiments in EM, showed that they contained aggregates both of complete particles and particles lacking the outer capsid, as well as empty particles.

To estimate the amount of virus needed to form a visible precipitin line in gel, a titration of a purified antigen as well as clarified faecal extracts were tested by IEOP A particle count was made on the same suspensions, using a Latex calibration method. The electrophoretic titre of the purified antigen was 1/128 after staining, while the clarified extracts gave titres of 1/64 and 1/16 There was a fourfold rise in all titrations after staining. The dilutions corresponded to about 3×10^7 particles/ml, which probably indicates the border line for a detection of reo-like virus by IEOP

DISCUSSION

The IEOP method used in the present work was found to be as sensitive as EM In contrast to findings in a preliminary study reported by *Spence et al.* (8) who out of 11 EM positive faecal extracts found only 5 positive extracts by IEOP If however a CF test was used for identification of virus in faecal samples, 10 out of the 11 were positive. The present authors have tried to repeat this identification by CF but the samples were often found to be anticomplementary In the first study where concentration of the faecal samples was necessary in 8 cases (13 per cent) the extracts often contained a lower percentage of faeces. These 8 samples were, according to the relative virus estimation in the former work (9) all + in the + to ++++ scale used. The low concentration of virus in many of the samples explains probably also the relatively low number of positive samples before staining in the first study The suspensions used in the second study all contained 20 per cent faeces and with this concentration 49 per cent of the EM-positive were detected before staining, while all were positive after staining The detection of virus by EM is, beyond doubt, a rapid test to be used whenever a diagnosis has to be established in the acute stage of illness. The performance of a number of tests, however is time consuming and requires expensive equipment. The IEOP on the other hand, is easily performed on a large scale, and it is extremely inexpensive as compared with the EM method. Even in the case of a relatively small amount of virus in the sample, which would require washing overnight and staining to make a precipitate visible, the IEOP-method seems preferable.

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PURIFICATION OF THE O ANTIGEN OF *BACTEROIDES FRAGILIS* SS *FRAGILIS* NCTC 9343 FROM PHENOL-WATER EXTRACTS BY GEL FILTRATION AND CHROMATOGRAPHY ON DEAE-CELLULOSE AND HYDROXYLAPATITE

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Hofstad, T Purification of the O antigen of *Bacteroides fragilis* ss. *fragilis* NCTC 9343 from phenol-water extracts by gel filtration and chromatography on DEAE-cellulose and hydroxyl apatite. Acta path. microbiol. scand. Sect. B, 84 229–234 1976.

O antigen extracted from whole cells of *Bacteroides fragilis* ss. *fragilis* NCTC 9343 with 45 per cent aqueous phenol has been purified by gel filtration and chromatography First, the water phase was treated with RNase and DNase and passed through a column of agarose. The chromatographic procedures included ion exchange on a column of DEAE-cellulose and adsorption to hydroxylapatite The O antigen was eluted from the DEAE-cellulose with a gradient of N Cl, and from the column of hydroxylapatite with 1 M phosphate buffer pH 6.8. Inhibition of indirect haemagglutination was used to detect the O antigen in the eluates.

Key words *Bacteroides fragilis* ss. *fragilis* O antigen purification gel filtration chromatography

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Lipopolysaccharides (LPS) isolated from *Bacteroides* species are unusual in several respects. They do not contain heptose or 2-keto-3-deoxy-octonate (KDO) (5) they have no typical lipid A (unpublished observations) and the endotoxic activity is low (4 7) They display however O-antigenic specificity Using haemagglutination and inhibition of haemagglutination at least three different specificities were detected in LPS isolated from *B. fragilis* ss. *fragilis* NCTC 9343 (6)

LPS purified from phenol-water extracts of NCTC 9343 and other *Bacteroides* strains by ultracentrifugation of the water phase are frequently contaminated with glucans (2, 6) rendering the preparation less suitable for immunochemical studies. The present paper describes an alternative procedure for purifi-

cation of the O-antigen of *B. fragilis* involving enzymatic treatment gel filtration ion exchange and adsorption chromatography

MATERIALS AND METHODS

Bacteroides fragilis ss. fragilis strain NCTC 9343 was grown in a chemostat at pH 7.0 and at dilution rates ranging from 0.05 to 0.11 h^{-1} (1). The medium was based on whale meat extract, and contained 0.6 per cent glucose as energy source and growth-limiting factor

Extraction and Purification Methods

Suspensions in distilled water of 20 mg acetone-dried cells or 200 mg packed, wet cells per ml were homogenized with equal volumes of 90 per cent phenol (15) for 15 min at room temperature (approx. 22 C)

The dialysed water phase was used immediately for preparation of LPS by ultracentrifugation (7) or kept frozen at −25 C after concentration by evaporation to approx. 1/10 of the original volume.

Digestion with ribonuclease (Ribonuclease-A, 5 × cryst., from bovine pancreas, Sigma Chemical Company St. Louis, Mo., USA) and deoxyribonuclease (Deoxyribonuclease I noncrystalline from bovine pancreas, Sigma Chemical Company) was performed in 0.1 M phosphate buffer pH 7.0. Bio-Gel A 1.5 M 100–200 mesh, agarose content 8 per cent (Bio-Rad Laboratories, Richmond, Calif., USA) was used for gel filtration. The column (4.5 × 85 cm) was stabilized and eluted with 0.1 M tris-HCl buffer pH 7.8, containing 0.001 M EDTA and 0.02 per cent sodium azide. The flow rate was 2 ml/cm^2h. Ion exchange chromatography was carried out on columns of DEAE-cellulose (DEAE-SS Serva, Heidelberg) Elution was performed with 0.02 M phosphate buffer pH 7.4 or with a NaCl gradient in the same buffer Column chromatography was also performed on hydroxylapatite (Bio-Gel HTP Bio-Rad Laboratories) equilibrated with 0.001 M phosphate buffer pH 6.8. The column (2.2 × 25 cm) was eluted step-wise with phosphate buffers, pH 6.8, of different molarities. Treatment with periodate was performed as described (3)

Serological Methods

Antisera were prepared in rabbits by immunization with whole NCTC 9343 cells (3)

Sensitization of sheep erythrocytes with N OH treated O antigen, the *indirect haemagglutination* (HA) technique *absorption* of antiserum with LPS and *inhibition of haemagglutination* have been described (6)

Endotoxic Activity

Primary skin inflammation was produced in white New Zealand rabbits (2 months old, 1.5 kg) (11) Graded doses (0.2 ml) of O antigen were injected subcutaneously on the lateral surfaces. Erythematous lesions, 5 × 5 mm or more present after 24 h, were recorded.

EXPERIMENTS AND RESULTS

In preliminary experiments, several preparations of LPS purified from phenol-water extracts of NCTC 9343 were examined for neutral sugars by gas liquid chromatography of acid hydrolysates. Rhamnose, fucose galactose, glucose and traces of mannose were detected in all preparations. The molar ratio of rhamnose to fucose was about the same, but the relative amount of galactose varied from one preparation to another All preparations contained large and varying amounts of glucose The same preparations were examined serologically for precipitating and erythrocyte-sensitizing activity and for the capacity to inhibit the agglutination of the sensitized sheep erythrocytes in rabbit antiserum to NCTC 9343 Weight by weight the preparations varied somewhat with respect to all three activities. There was, however no parallelism in the variations of precipitating activity against NCTC 9343 antiserum and inhibition of haemagglutination.

The results of the preliminary experiments thus indicated that, besides glucans, the conventionally prepared NCTC 9343 LPS might contain precipitating antigens not necessarily part of the O antigen. Other experiments disclosed the presence in the supernatant fluid following ultracentrifugation of considerable amounts of sensitizing antigen. Attempts were made, therefore to isolate the erythrocyte-sensitizing substance i.e. the O antigen, directly from the dialysed water phase. A procedure including treatment of the water phase with ribonuclease and deoxyribonuclease, filtrations through Bio-Gel A 1.5 m, ion exchange with DEAE-cellulose and adsorption to hydroxylapatite, was finally adopted (Fig 1) Part of the procedure had previously been used for isolation of LPS

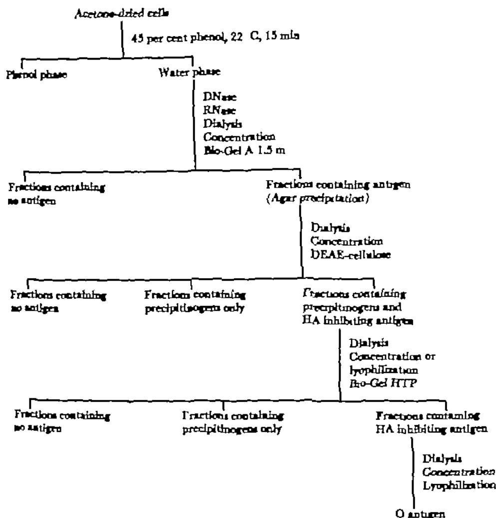


Fig 1 Preparation of O antigen from *B. fragilis* ss. *fragilis* NCTC 9343

from the supernatant fluid following ultracentrifugation of the water phase from phenol-water extracts of *Bacteroides melaninogenicus* and *Fusobacterium necrophorum* (*Sphaerophorus necrophorus*) (8 9)

In a large-scale experiment, concentrated and frozen water phase (20 ml) from extraction of 10 g acetone dried NCTC 9343 organisms was thawed at room temperature, mixed with 4 mg ribonuclease and 4 mg deoxyribonuclease in 60 ml of buffer and incubated at 37 C for 2 h. Following dialysis overnight against tap water and concentration in a rotary evaporator to approx. 40 ml the digest was applied to the Bio-Gel A 1.5 m column. 15 ml fractions were collected and examined by double diffusion in agar against antiserum NCTC 9343 (Fig 2) Preliminary experiments had shown that the erythrocyte-sensitizing substance was eluted in the same fractions as the precipitating antigens. All antigen-containing fractions were pooled, dialysed against tap water and the volume reduced by evaporation. The concentrated pool was mixed with antigen-containing eluates from Bio-Gel A 1.5 m obtained in the same way from extracts of 3 other 10 g batches of NCTC 9343 organisms. A small sample was freeze-dried (crude antigen) The rest was applied to a 4.5 cm wide and 60 cm

15*

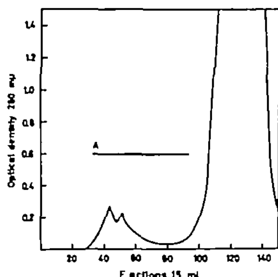


Fig. 2. Gel filtration of DNase- and RNase-treated water phase on a 85 cm high column of Bio-Gel A-1.5 m with diameter of 4.5 cm. A: Serologically active material.

high column of DEAE-cellulose. The column was eluted with buffer until no more ultra violet light absorbing material appeared in the fractions (Fig. 3). Thereafter a linear NaCl gradient in the buffer was used for elution. The fractions were examined by double diffusion in agar and inhibition of haemagglutination in 4 units of antiserum NCTC 9343. The sheep erythrocytes were sensitized with 12.5 μg/ml of crude antigen.

As measured by haemagglutination inhibition, some sensitizing antigen went straight through the column together with precipitinogens, but most of it was eluted with 0.05 to 0.4 M NaCl. The fractions were pooled as indicated in Fig. 3 (pools I–V), dialysed against tap water and concentrated by evaporation. Pools II, IV and V were each subjected to a new run on DEAE-cellulose (column dimensions 3.5 × 56 cm). As shown in Fig. 4 the material in pool II inhibiting haemagglutination was now separated nearly completely from the precipitating antigen. Likewise, some fractionation of sensitizing and precipitating antigens in pool IV but not in pool V was achieved by repeated ion exchange chromatography. The fractions from all three DEAE-cellulose runs which contained material inhibiting the haemagglutination were pooled, dialysed against tap water and freeze-dried. An unknown amount of the material was accidentally lost during freeze-drying. 117 mg of the recovered material (170 mg) were suspended in distilled water and applied on the Bio-Gel HTP column. A nearly complete separation of precipitating and sensitizing antigens, as judged by double diffusion in agar and inhibition of haemagglutination, was achieved (Fig. 5). Finally the fractions inhibiting haemagglutination were pooled, dialysed against tap water followed by dis-

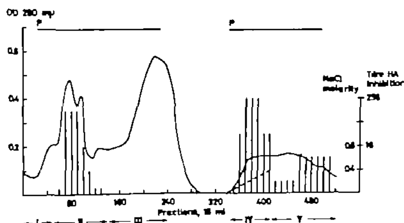


Fig. 3. Chromatography of serologically active material from Bio-Gel A 1.5 m (Fig. 2) on 60 cm high column of DEAE-cellulose with a diameter of 4.5 cm. Bars: Titre of fractions in HA inhibition. P: Precipitating antigens.

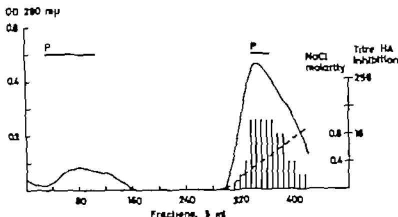


Fig. 4. Chromatography of pool II Fig. 3 on a 3.5 cm wide and 56 cm high column of DEAE-cellulose. Bars: Titre of fractions in HA inhibition. P: Precipitating antigens.

tilled water and freeze-dried. The yield of freeze-dried material (i.e. O antigen) was 32 mg.

The endotoxic activity of the prepared O antigen was compared with that of NCTC 9343 LPS, purified by ultracentrifugation, in two rabbits. Both preparations produced primary skin lesions in doses down to 25 μg.

DISCUSSION

Gel filtration alone or in combination with chromatography on DEAE-cellulose has previously been used for isolation of LPS from the supernatant fluid of cultures of *Escherichia coli* (12) and *Shigella sonnei* (14) and from phenol water extracts of *S. sonnei* (13) and *Fusobacterium necrophorum (Sphaerophorus necrophorus)* (9). The theoretical basis for purification of LPS by these methods is that LPS are macromolecules with anionic properties. The elution profiles of the O-antigenic material of NCTC 9343 on Bio-Gel A 1.5 m and DEAE-cellulose indicate that the O-antigen of *B. fragilis* like the *Salmonella* O antigens, constitute the antigenically

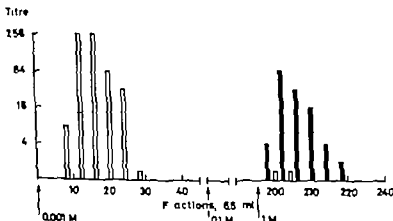


Fig. 5. Chromatography of antigenic material (HA inhibition) recovered after rechromatography of pools II, IV and V Fig. 3 on a 25 cm high column of Bio-Gel HTP with a diameter of 2.2 cm. The column was eluted stepwise with phosphate buffer pH 6.8. Open bars: Titres of fractions in precipitation. Closed bars: Titres of fractions in HA inhibition.

active part of the endotoxic LPS complex. This assumption is in agreement with earlier serological studies (6) and is supported by the finding in rabbits of an endotoxic activity of the isolated material.

A further purification of the O antigen of NCTC 9343 was obtained by chromatography on hydroxyl apatite. Most likely the affinity to the hydroxyl apatite was due to interactions between negatively charged (phosphate?) groups of the O-antigenic LPS complex and the positive charges of calcium ions in the sorbent crystals.

The results of the gel filtration and ion exchange chromatography suggest that the O-antigenic material is heterogenous with respect to molecular weight and electrical charge. *Kent & Osborne* (10) have shown that free O-specific haptens isolated from *Salmonella typhimurium* are weakly anionic and bind reversibly to DEAE-cellulose. If present in phenol/water extracts of NCTC 9343 free haptens may have been isolated together with the O-antigenic LPS polymers.

Preliminary chemical examinations have indicated that the purified O antigen is not contaminated with glucans and is nearly protein-free. The fractionation procedure, therefore, seems to be well suited for preparation of O antigen for immunochemical stud-,, and also for isolation of neutral polysaccharide antigens. The drawback is that it is a rather laborious and time-consuming method and the O antigen is sometimes obtained in small yields.

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A TECHNIQUE FOR OBTAINING THIN SECTIONS OF COCCIDIAN OOCYSTS

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Birch-Andersen, A., Ferguson, D J P & Pontefract, R. D A technique for obtaining thin sections of coccidian oocyst. Acta path. microbiol. scand. Sect. B, 84 235–239 1976

A double sectioning technique is described for obtaining thin sections of coccidian oocysts. This method employs cryostat sectioning of unfixed oocysts prior to treatment by normal methods of fixation, dehydration, embedding and thin sectioning for electron microscopy A number of the oocysts treated by this technique were well preserved and contained organelles with normal ultrastructure.

Key words Ultrastructure coccidian oocysts; cryostat thin sections.

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In order to survive and propagate, parasites must pass from one host to another and during this transitional period leave the protected environment of the host. To facilitate this they may use a transient adaptation to the internal milieu of an intermediate host, or they may produce resistant forms. To withstand the rigors of the external environment these resistant forms normally possess a protective coat or wall.

The group coccidia, for example, contains many parasites which do produce resistant forms called oocysts which are protected by an impervious wall (Fig. 1) Hitherto the ultrastructure of oocysts of coccidian species has not been described because of the difficulty in preparing sections of them for examination with the transmission electron microscope. During our attempts to produce suitable preparations of such oocysts we found that, irrespective of the fixative (glutaraldehyde, formaldehyde acrolein) or combination of fixatives used, the cytoplasmic mass within the oocyst was poorly fixed and collapsed when dehydrated. In addition because of the impermeability of the oocyst wall, poor embedding was obtained with Vestopal W and Spurr's low viscosity Epon (*Spurr* 1969) (Fig 2)

These difficulties were overcome when a double sectioning technique was developed involving cryostat sectioning of rapidly frozen oocysts prior to chemical fixation and resin embedding. The method is described in the present paper and examples of the preservation of ultrastructural details are given. The

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oocysts of *Eimeria brunetti* were used as our model in these experiments.

MATERIALS AND METHODS

Oocysts of *E. brunetti* were obtained from the faeces of infected chickens The oocysts were con centrated using a salt flotation technique employ ing zinc sulphate This process was carried out at 4 C using pre-cooled reagents and an RC-3 Sorvall centrifuge maintained at 4 C. Sporulation was prevented by storing the oocysts in 2 per cent potassium dichromate at 4 C.

Fixation and Embedding The oocysts were washed in deionised water and densely packed by centrifugation into a sol tion consisting of 20 per cent (w/v) Bovine Serum Albumin (BSA) and 15 per cent (w/v) sucrose in phosphat buffered saline (PBS) pH 7 4 The supernatant was discarded and the pellet solidified by cross-linking the BSA with glutaraldehyde. To accomplish this 3 drops of 25 per cent glutaraldehyde per 1 ml of pellet was usually sufficient to yield a block firm enough for further processing. The block so obtained was soaked in 30 per cent sucrose for 1 hour prior to being placed on a cryostat chuck and quick frozen in either Freon 22 cooled to its melt ing point by liquid nitrogen or in super cooled liquid nitrogen (*Umrath* 1974) The block was sectioned at the 20 µm setting of an Ames Lab-Tek cryostat with knife and block temperatures adjusted to between 20° and –30 C. The cryostat sections were quickly transferred and allowed to thaw in Karnovsky fixative (*Karnovsky* 1965) prepared with cacodylate buffer pH 7.2 and 0 01 M with respect to calcium chloride Fixation was carried out at room temperature for a minimum of 5 hours. The sections were washed by repeated centrifugation in PBS and finally soaked overnight in thi sol tion The sections were then re-embedded in 20 per cent BSA, this time without sucrose, but solidified by the addition of glutaraldehyde a before The block thus obtained was cut into 1 mm³ cubes, which were postfixed in 1 per cent osmium tetroxide in eronal acetate buffer pH 7.3, for 1 hour and treated *en bloc* with 2 per cent uranyl acetate in eronal acetat buffer for another hour The cubes were dehydrated through a graded ethanol series and embedded in Vertopal-W after propylene oxide treatment. Sections were cut with glass knives on an LKB microtome, stained with magnesium uranyl acetate (*Frasca & Parks* 1965) and lead citrate (*Reynolds* 1963) and examined with a Philips EM 200 electron microscope.

RESULTS AND DISCUSSION

By cryostat sectioning of the oocysts it was possible to cut or fracture the impervious oocyst wall, and thus allow the fixing and embedding agents access to the interior of the oocyst. The ultrastructural details were preserved from ice crystal damage by the extremely quick freezing of the oocysts and by using sucrose as a cryo-protectant. Further more thawing the cryostat sections directly in the fixative provided a primary fixation after which the use of standard procedures for preparation of thin sections was possible. However the actual thickness of the cryostat sections prepared was found to be important because if the sections were too thick, many of the oocysts were unopened and thus impermeable and if too thin, many of the cytoplasmic masses within the oocysts were destroyed when hit by the rather coarse knife edge The oocysts of *E. brunetti* have an average size of approximately 27 µm by 22 µm and it was found that a section thickness of 20 µm gave the greatest yield of well-preserved material If the technique is to be applied to oocysts of a different size then the optimal cryostat sectioning thickness will have to be adjusted accordingly

Fig 1 A phase contrast micrograph of an unsporulated oocyst showing the cytoplasmic mass enclosed by the oocyst wall. × 650

Fig 2 An electron micrograph of an oocyst showing the poor preserv tion obtained with normal preparative procedures. Note the collapsed and poorly embedded cytoplasmic mass. × 1440

Fig 3 An electron micrograph of an unsporulated oocyst obtained by the technique described showing the well preserved cytoplasmic mass. × 7500.

Fig 4 An enlargement of the cytoplasmic mass (a) of Fig 3 showing the rough endoplasmic reticulum (ER) and a Golgi body (G) × 90000

Fig 5 An enlargement of the cytoplasmic mass (b) of Fig. 3 showing the limiting unit membrane (L) a mitochondrion (M) rough endoplasmic reticulum (ER) and the nucleus (N) × 90000.

We are indebted t the Central Veterinary Laboratory Ministry of Agriculture, Fisheries, and Food, New Haw Weybridge, Surrey England, for supplying the pure sample of oocysts of *E. brunetti* used to infect the chickens.


2
3
b
G
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M
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5

The use of BSA as an embedding medium has two advantages

1 Both the oocysts and the prefixed cryostat sections, which were soaked in BSA, could be centrifuged into concentrated pellets prior to solidification by the glutaraldehyde.

2 Crossed linked BSA, in comparison to Agar for example, can withstand freezing and thawing and is thus an ideal medium for cryostat sectioning. After fixation, dehydration, and embedding it is possible to obtain reasonable results using the standard methods of ultramicrotomy

In the first BSA embedding used to obtain blocks for the cryostat, sucrose was added as a cryo-protectant. It was found advantageous to omit the sucrose in the second BSA embedding because it made the plastic embedded blocks brittle and difficult to section with the ultramicrotome.

When the sections obtained by this technique were examined after staining with toluidine blue, it was found that although many of the oocysts had been destroyed by the cryostat knife, a reasonable number were well preserved. At the ultrastructural level it was observed that within the oocyst wall there was a cytoplasmic mass limited by a delicate unit membrane (Figs. 3 4 & 5) The cytoplasm contained a number of easily identifiable organelles such as polysaccharide granules, mitochondria with bulbous cristae Golgi bodies, vesicles of various sizes, varying amounts of smooth and rough endoplasmic reticulum, free ribosomes and a well preserved nucleus (Figs. 3 4 & 5) Also, the cytoplasmic matrix appeared well preserved and the structure of the organelles was similar to the ultrastructure of the same organelles of the macrogamete as observed in the intestine of the chicken (*Ferguson et al.* in prep.)

The preparative method outlined here is quite similar to one recently published by *Gardner et al.* (1975) for the study of fungal spores. We were unaware of the methods employed by this group when our own experiments were carried out and there are certain points where the two methods differ In our procedure there is no need for chemical fixation prior to embedding in BSA and cryostat sectioning. In addition, we used a standard cryostat, not a cryo-ultramicrotome.

The double sectioning technique described in this paper is now being applied to study the ultrastructural changes occurring as the oocysts of *Eimeria spp* and *Toxoplasma gondii* develop (sporulate) This is being done by examining oocysts which have been allowed to sporulate for different time intervals.

Recently *Dubremetz et al.* (1975) reported on the possible use of the freeze etching method to study the ultrastructure of coccidian oocysts. However the method described here is advantageous compared to freeze etching because the results are easier to interpret and can be compared directly to the ultrastructure of other stages in the life cycle where standard methods for ultramicrotomy and section staining are being used. With this method it is also possible to obtain serial sections which can provide information on the organelle distribution within the unsporulated and sporulated oocysts.

This work is a part of a project on the life cycle and biology of *Toxoplasma gondii* which is carried out as a co-operation between Professor *W M Hutchison* Department of Biology University of Strathclyde, Glasgow Scotland, and Dr *J Chr Siim* Department of Toxoplasmosis, Statens Seruminstitut Copenhagen, Denmark.

This project is supported by grants from the *World Health Organisation* Geneva *The Wellcome Trust* and the *British Council*.

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NDV-O AGGLUTININS IN *MYCOPLASMA PNEUMONIAE* INFECTIONS ASSOCIATION WITH PNEUMONIA

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Pyhälä, R. NDV-O agglutinins in *Mycoplasma pneumoniae* infections: association with pneumonia. Acta path microbiol scand. Sect. B, 84: 240 24 1976

Agglutinins against human group O erythrocytes modified by the B1 strain of Newcastle disease virus were studied in paired sera of 148 patients with a 4-fold or greater rise in complement fixing *M pneumoniae* antibodies. The proportion of cases with a significant rise in NDV-O agglutinins was higher among the patients with pneumonia than among those with neurological or other clinical manifestations.

Key words *Mycoplasma pneumoniae* infections NDV-O agglutinins pneumonia.

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Increased titres of agglutinins against red blood cells (RBC) modified by Newcastle disease virus (NDV-O agglutinins) are found in several infectious diseases. Such rises are frequent in infectious mononucleosis (3) and in infection with *Mycoplasma pneumoniae* (1) The agglutinins in these two infections differ serologically (18) those formed in *M pneumoniae* infection being related to the T agglutinins detected with RBC modified by neuraminidase of *Vibrio cholerae* (17) Elevated T agglutinin titres were demonstrated by *Lind* & *McArthur* (13) in a high proportion of patients with atypical pneumonia. The occurrence of cold agglutinins in the majority of these cases suggests that the disease was caused by *M pneumoniae*

In recent years *M pneumoniae* has been one of the agents most commonly responsible for certain respiratory infections, e.g pneumonia in Finland (7 19) as well as in the other Scandinavian countries (15 16) Significant rises in antibodies against *M pneumoniae* antigens have also been observed more or less frequently among patients with other than respiratory symptoms, e.g neurological manifestations (9 10 15 16 21) In the present study the occurrence of NDV-O agglutinins was analysed in relation to certain clinical findings and to the age in the patients, who showed a significant rise in antibodies against *M pneumoniae*

MATERIALS AND METHODS

Paired sera from 148 patients showing 4-fold or greater rise in complement fixing (CF) antibodies against *M pneumoniae* were selected for study among specimens sent from general hospitals to the Department of Virology Central Public Health Laboratory Finland, in 1974–75 One specimen had been taken in the acute and the other in the

convalescent phase of the disease. Specific data on the occurrence of pneumonia and neurological manifestations as well as the final clinical diagnosis were obtained through a questionnaire sent to the attending physicians when the sera had been studied for CF antibodies. This information was used for grouping the patients into three categories, later termed clinical groups

A Patients with pneumonia (40 cases)
B Patients with neurological involvement but no evidence of pneumonia (39 cases) Among these, the diagnosis was meningitis in 25 meningoencephalitis in 8, encephalitis in 5 and Guillain-Barré syndrome in one.
C A miscellaneous group without lower respiratory infections or central nervous system disease (69 cases) The majority of the patients had had upper respiratory tract infection or a febrile illness with non-specific symptoms. In addition, the group included patients with the following main findings pericarditis (9 cases) gastroenteritis (4 cases) a skin and mucocutaneous disorder (6 cases) pancreatitis (3 cases) and arthritis (1 case)

Each of the three groups was further subdivided by age into two groups, 30 years being the limiting value.

The CF antibodies against *M. pneumoniae* were

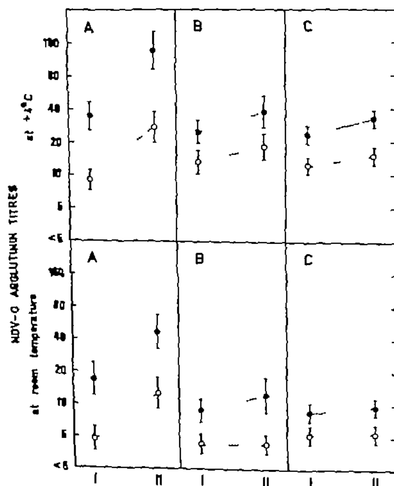


Fig 1 Geometric mean titres (± S.E.) of NDV-O agglutinins in acute- (I) and convalescent-phase (II) sera of patients showing a significant rise in antibodies against *M. pneumoniae*. A = Patients with pneumonia. B = Patients with neurological manifestations. C = Patients with miscellaneous manifestations. For details see Materials and Methods. White circles = Patients >30 years old. Black circles = Patients ≤30 years old.

determined with a microtitre technique (4) using 2 full units of complement, 4 units of antigen and a 2.5 per cent suspension of sensitized sheep RBC. Fixation was allowed to proceed overnight. The antigen obtained from a commercial source (Orion, Helsinki, Finland) had been prepared as described by *Kenny & Grayston* (8).

In five of the six groups, the geometric mean titre of *M. pneumoniae* antibodies in the acute phase ranged from 7 to 11. In the group of younger pneumonia patients the titre reached 19. The average rises in antibody titre in the six groups ranged from 7.0-fold to 9.4-fold. The lowest rise was recorded in the younger patients with neurological manifestations and the highest in the older patients with miscellaneous symptoms.

The paired sera were studied for NDV-O agglutinins in random order without knowledge of diagnosis or age. Before the determination the sera were absorbed with unmodified RBC at +4 C.

RBC from healthy group O donors were modified, as presented previously (1), with the B1 strain of NDV grown in embryonated eggs. The haemagglutinin titres of the virus-infected allantoic and amniotic fluids, determined with chicken RBC, ranged from 512 to 1024. The modified RBC were used immediately to test the sera for NDV-O agglutinins at room temperature and at +4 C. The procedure of a microtitre method described previously (1) were followed.

RESULTS

The geometric mean titres (GMT) of NDV-O agglutinins determined at +4 C and at room temperature are shown by age and clinical groups in Fig. 1. The agglutinin level was higher at +4 C than at room temperature. In other respects the results at +4 C did not differ substantially from those obtained at room temperature. In all three clinical groups, in both the acute and the convalescent phase, the GMT was higher in the younger patients (aged ≤30 years). A statistically significant rise in GMT was found only in group A, the pneumonia patients ($P<0.01$).

The distribution of patients with a 4-fold or higher rise in NDV-O agglutinins by clinical group and age group is given in Table 1. In each of the six groups the proportion was higher when the agglutinins were determined at +4 C. The proportion was highest among pneumonia patients under 30 years and lowest in the group of patients with miscellaneous symptoms and over 30 years old. The latter clinical group, when patients of all ages are taken into consideration, included only 8 serum pairs that showed a significant rise in NDV-O agglutinins. In two of them the main finding was gastroenteritis and in the others an upper respiratory infection. Thus, significant rises were not demonstrated in connection with the other symptoms listed in Materials and Methods.

TABLE 1. *Proportion of Cases with a 4-fold or Greater Rise in NDV-O Aggl tinins among Patients Showing Significant Rise in Antibodies against* M. pneumoniae

Clinical group	Age group	Agglutinins determined at +4 C	+22° C
A	≤30 years	10/19 (53%)	9/19 (47%)
	>30	9/21 (43%)	7/21 (33%)
B	≤30	6/26 (23%)	5/26 (19%)
	>30	2/13 (15%)	0/13 (0%)
C	≤30	6/41 (15%)	1/41 (2%)
	>30	2/28 (7%)	0/28 (0%)

A = Patients with pneumonia.
B = Patients with neurological manifestations.
C = Patients with miscellaneous manifestations.
For details see Materials and Methods.
Significance of difference when agglutinins are determined at +4 C:
Groups A and B $\chi^2 = 5.24$ $P<0.025$.
Groups A and C $\chi^2 = 15.63$ $P<0.001$

DISCUSSION

In a previous study on *M. pneumoniae* patients (1), the occurrence of NDV-O agglutinins and cold agglutinins (CA) with anti-I specificity was shown to be associated with young age. The analysis by age groups suggested further association between these agglutinins. The CA of *M. pneumoniae* patients have been reported to be directly related to the severity of the illness and the involvement of the lungs (2, 11). In patients with neurological symptoms the proportion of CA positive cases has been reported to be similar to the overall frequency in patients with respiratory infections (10). In the present study the NDV-O agglutinins were associated

with certain clinical findings in a way which seems to be somewhat analogous to the above-mentioned results on CA. It was not possible, however to study the present series for CA, owing to lack of serum or separation at unsuitable temperatures.

There are several possible explanations for the more frequent development of NDV-O agglutinins in pneumonia patients. 1) The antigenic stimulus leading to the production of NDV-O agglutinins might be characteristic of patients with pneumonia, regardless of the etiology of the disease. This seems to be ruled out, for in the series studied previously (1) there were numerous patients with pneumonia caused by agents other than *M pneumoniae* who did not show any rise in NDV-O agglutinins. 2) The NDV-O agglutinins might be cross-reacting antibodies evoked by *Mycoplasma* organism or might result indirectly from *M pneumoniae* infection. If so, it is possible that some of the NDV-O agglutinin-negative patients, especially those without symptoms of pneumonia, do not represent cases of true *M pneumoniae* infection. Indeed, it has been proposed that in patients with pancreatitis the significant rises in *M pneumoniae* antibodies are not evoked by this organism (9) 3) The antigenic stimulus responsible for production of NDV-O agglutinins during *M pneumoniae* infection might operate mainly in patients with lung involvement.

In the two latter instances the antigen which triggers off NDV-O agglutinin production may be a component of the *M pneumoniae* organism, as suggested in connection with CA (6, 12) Another possibility is that *M pneumoniae* acts on some hidden antigen present in the cells of the patients, rendering it autoantigenic. The NDV-O agglutinin produced by *M pneumoniae* patients has been shown to be directed against a latent RBC receptor T which is activated by treatment with neuraminidase (17) Colonies of *M pneumoniae* are known to attach to RBC and to tracheal epithelial cells by neuraminic acid receptors on the cells (5 14 20) This attachment may provide the *M pneumoniae* organism with an opportunity to uncover autoantigenic groupings on the membranes of tissue cells. Studies on the different possibilities are in progress.

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RAPID DIAGNOSIS OF *ENTEROBACTERIACEAE*

I Detection of Bacterial Glycosidases

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Kilian, M. & Bülow P Rapid diagnosis of *Enterobacteriaceae* I Detection of bacterial glycosidases. Acta path. microbiol scand. Sect. B, 84 245-251 1976.

The paper describes a number of tests for the rapid detection of glycosidases including α-glucosidase β-glucosidase, β-glucuronidase, β-xylosidase and α-fucosidase. The methods use heavy suspensions of viable but non-multiplying bacteria in a buffered solution of a chromogenic substrate. The results of the tests are readable within 4 h. The application of these tests to a collection of 633 strains of *Enterobacteriaceae* and *Vibrionaceae* demonstrates that some of the tests may be valuable additions to the present tests available for the identification of bacteria belonging to these families. β-glucuronidase activity was observed only in strains of the *Escherichia-Shigella* group 97 per cent of the *Escherichia* strains possessed β-glucuronidase activity β-xylosidase activity was almost completely restricted to strains of the *Klebsiella-Enterobacter* group in addition to *Yersinia* strains. None of the strains possessed α-fucosidase activity

Key words *Enterobacteriaceae* *Vibrionaceae* rapid identification glycoside hydrolases.

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Chromogenic enzyme substrates are used widely in biochemistry for the purpose of studying kinetics of enzyme activities. In bacterial characterization, this kind of substrate has been applied in the ONPG test (3 9 11 12). This test, based upon the demonstration of the coloured nitrophenol released from the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) by the activity of enzymes formed by suspensions of bacteria allows a rapid detection of β-galactosidase. Whenever possible it seems desirable that bacterial characterizing tests should be carried out in a similar manner The interpretation of results obtained by such tests are not complicated by side effects of the multiple reactions that may occur in complex nutrient culture media containing the substrate.

Chromogenic synthetic substrates which allow detection of a variety of enzyme reactions are now commercially available The present paper describes a number of methods for the rapid detection of bacterial glycosidases using nitrophenyl glycopyranosides. The application of these tests to a collection of 633 strains of *Enterobacteriaceae* and *Vibrionaceae* demonstrates that some of the tests may be valuable additions to the present tests available for the identification of these groups of bacteria.

MATERIALS AND METHODS

Bacterial Strains

The 633 strains distributed according to species and sources are listed in Table 1. Of these strains, 448 were freshly isolated from clinical samples and were identified by conventional methods (5, 6). The remaining strains were received from the culture collection of the Diagnostic Department, Statens Seruminstitut, Copenhagen (Drs. *H. Lautrop* and *K. Gaarslev*) and from the National Collection of Type Cultures, Colindale, London. The *Shigella dysenteriae* strains included three strains of serotype 1 (NCTC 4837, NCTC 6781, NCTC 9718), two strains of serotype 3 (NCTC 9730, SSI 31883), and one strain of each of the sero-

TABLE 1. *Designation and Source of 633 Strains Tested*

Designation	No. of strains	Source		
		Fresh isolates	SSI	NCTC‡
Aeromonas formicans	8	–	8	–
Aeromonas hydrophila	10	–	10	–
Citrobacter freundii	10	10	–	–
Citrobacter koseri	7	7	–	–
Edwardsiella tarda	6	–	6	–
Enterobacter aerogenes	29	24	5	–
Enterobacter cloacae	36	36	–	–
Escherichia adecarboxylat	3	–	3	–
Escherichia coli	109	109	–	–
Escherichia dispar	2	2	–	–
Escherichia alkalescens	2	1	–	1
Erwinia herbicola	7	–	7	–
Hafnia alvei	10	–	10	–
Klebsiella oxytoca	50	50	–	–
Klebsiella ozaenae	5	–	5	–
Klebsiella pneumoniae	135	135	–	–
Klebsiella rhinoscleromatis	10	–	10	–
Proteus inconstans	9	9	–	–
Proteus mirabilis	19	19	–	–
Proteus morgani	25	25	–	–
Proteus rettgeri	7	1	6	–
Proteus vulgaris	7	7	–	–
Salmonella typhi		2	–	–
Salmonella, different serotypes	8	8	–	–
Serratia liquefaciens	10	1	9	–
Serratia marcescens	14	2	12	–
Serratia rubidaea	10	–	10	–
Shigella boydii	5	–	3	2
Shigella dysenteriae	13	–	1	12
Shigella flexneri	13	–	13	–
Shigella sonnei	14	–	14	–
Vibrio alginolyticus	10	–	10	–
Vibrio cholerae	11	–	11	–
Vibrio parahaemolyticus	1	–	1	–
Yersinia enterocolitica	10	–	10	–
Yersinia pestis	1	–	–	1
Yersinia pseudotuberculosis	5	–	5	–
Total	633	448	169	16

SSI: Statens Seruminstitut, Copenhagen (Drs. *H. Lautrop* and *K. Gaarslev*)
‡ NCTC: National Collection of Type Cultures, Colindale, London.

types 2 and 4–10 (NCTC 9719 NCTC 9759 NCTC 9721 NCTC 9762, NCTC 9763 NCTC 9345, NCTC 9347 and NCTC 9351 respectively). The five strains of *Sh. boydii* included the strains NCTC 9323 (serotype 1) and NCTC 973 (serotype 2). The *Yersinia enterocolitica* strains included seven strains of serotype 3 and three strains of serotype 9. One of the strains of *Escherichia alkalescens* was NCTC 9775; the *Aeromonas hydrophila* strains included ATCC 11163 (*A. punctata*) and ATCC 13442; the strain of *Vibrio parahaemolyticus* was ATCC 17802, the *V. alginolyticus* strains included strain ATCC 17749 and the strain of *Yersinia pestis* was NCTC 5923 (serotype 1). The five strains of *Yersinia pseudotuberculosis* included a strain of each of the serotypes 1 to 5.

Cultural Conditions

The bacteria were cultured on nutrient agar (Statens Seruminstitut) incubated at 35 °C. The few strains that were unable to grow satisfactorily on this medium were cultured on horse blood agar (5 per cent v/v) or chocolate agar. For the quantitative enzyme assays, the bacteria were harvested from the agar plates and washed twice in 0.85 per cent (w/v) saline before being suspended in the enzyme substrates.

Determination of pH Optima

The chromogenic enzyme substrates (Table 2) were dissolved in 1/15 M phosphate buffers (Sørensen) pH 6.0, 6.5, 7.0, 7.5, 8.0 and 0.2 M Tris/HCl buffers, pH 8.0, 8.5, 9.0. A reaction mixture containing the enzyme substrate and a standardized quantity of washed bacteria (about 2 mg dry weight per ml) was incubated at 35 °C. The bacteria were removed after 4 hours by centrifugation at 10,000 g for 10 min at 4 °C in a Sorvall centrifuge model RC-2. The enzyme activities were estimated by released nitrophenol in the supernatants. The light absorption of 2-nitrophenol and 4-nitrophenol was determined at 430 μm and 400 μm, respectively using a Spectronic 20 photometer (Bausch & Lomb). In a duplicate set of reaction mixtures, the reactions were terminated after removing the bacteria by the addition of 1 ml. of 0.5 M carbonate-bicarbonate buffer pH 10.6. The colour was measured as described above.

In the pH-assays, one strain of each of the following species was used: α-glucosidase: *Escherichia coli*; β-glucosidase: *Klebsiella pneumoniae*; β-glucuronidase: *Escherichia coli*; β-xylosidase: *Enterobacter cloacae*; and α-fucosidase: *Haemophilus paragallinarum* (strain HK 364).

Standard Procedure

For the standard routine procedure applied to all strains listed in Table 1, one loopful of bacteria (about 1 mg dry weight) was suspended directly in 0.5 ml of the enzyme substrates (Table 2) contained in a Widal test tube. The test for β-galactosidase (ONPG) was performed as described previously (3).

The development of a yellow colour indicating released nitrophenol was recorded after incubation at 35 °C for 4 hours.

RESULTS

The glycosidase reactions studied had pH optima between 7.0 and 8.0. These values were determined colorimetrically after addition of carbonate-bicarbonate buffer. The optimal colour reactions as determined without preceding addition of carbonate-bicarbo-

TABLE 2. *Composition of Glycosidase Test Substrates*

Enzyme	Substrate	Concentration (w/v)	Buffer	pH	Abbreviation used
α-glucosidase 3.2.1.20	4-Nitrophenyl-α-D-glucopyranoside (Merck)	0.1 %	Phosphate (Sorensen) 1/15 M	8.0	PNPG
β-glucosidase 3.2.1.21	4-Nitrophenyl-β-D-glucopyranoside (Merck)	0.1 %	Phosphate (Sorensen) 1/15 M	8.0	NPG
β-glucuronidase 3.2.1.31	4-Nitrophenyl-β-D-glucopyranosiduronic acid (Merck)	0.1 %	Tris/HCl 0.2 M	8.5	PGUA
β-xylosidase 3.2.1.37	2-Nitrophenyl-β-D-xylopyranoside (Koch Light)	0.1 %	Phosphate (Sorensen) 1/15 M	8.0	ONPX
α-fucosidase 3.2.1.51	2-Nitrophenyl-α-L-fucopyranoside (Koch-Light)	0.1 %	Phosphate (Sorensen) 1/15 M	7.5	ONPF

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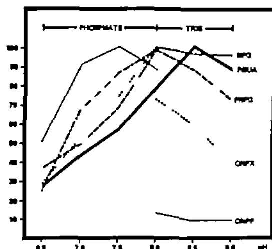


Fig 1 Effect of pH on the release of nitrophenol from
4-nitrophenyl-β-D-glucopyranoside (NPG)
4-nitrophenyl-β-D-glucopyranosiduronic acid (PGUA)
4-nitrophenyl-α-glucopyranoside (PNPG)
2-nitrophenyl-β-D-xylopyranoside (ONPX) and
2-nitrophenyl-α-L-fucopyranoside (ONPF)
Values plotted as percentage of maximal extinction.

nate buffer were about 0.5 pH units higher (Fig 1) Accordingly the final enzyme substrate solutions (Table 2) were prepared at the higher pH.

The activities of β-xylosidase (ONPX) and α fucosidase (ONPF) were inhibited in Tris/HCl buffer (Fig 1)

Storage of the enzyme substrates under various conditions revealed varying degrees of non specific break-down of the nitrophenyl glycopyranosides. When exposed to light, a yellow colour developed within a few days. However the substrates were stable for several months at 4 C as well as at 35 C when stored in the dark.

The glycosidase activities of the 633 strains tested are shown in Table 3 The results listed were obtained after 4 hours incubation. None of the strains possessed detectable α fucosidase activity β-glucuronidase activity was observed only in strains of the *Escherichia-Shigella* group. In contrast, β xylosidase activity was almost completely restricted to strains of the *Klebsiella-Enterobacter* group in addition to *Yersinia* strains.

The majority of reactions were readable after 2 to 3 hours. However with the exception of *Yersinia* strains, the intensity of the colour reactions seen in the α-glucosidase test was always weak compared to reactions in *other substrates. The β-glucosidase test, in* addition to negative and strong positive reactions, revealed weak intermediary colour intensities. The intensity of the reaction was characteristic of the individual species. In contrast to the other tests, weak reactions in the two glucosidase substrates could usually not be intensified by using a larger inoculum and/or by prolonging the incubation.

DISCUSSION

The principle of the glycosidase tests described above is identical to that applied in the well-established ONPG tests for β-galactosidase (3 12) In these tests, induced enzymes from heavy suspensions of viable but non multiplying bacteria act on a buffered solution of a chromogenic substrate. As a result of the enzyme activity a yellow product (2 or 4-nitrophenol) is released. The advantage of this principle is that the bacteria act only on one substrate in a test. Strong enzyme reactions were obtained without inducers added to the growth medium used for inoculum cultures. The ONPG and ONPX tests have been used previously in a modified method using paper discs impregnated with the substrate (2, 4) The application of these discs on top of the growth on a complex agar medium, however inevitably *involve some of the disadvantages of the* conventional bacterial characterizing tests.

The tests were designed to give an optimal and unambiguous colour reaction within 4 hours of incubation. To obtain this, the density of the suspensions used in the tests was about 2–3 × 10 cells/ml, below which the reactions were slower and less certain. The significantly reduced activities of some enzymes observed in Tris-buffer emphasize the importance of a suitable buffer The pH values chosen for the final substrates were those giving an optimal colour reaction As the

TABLE 3 *Glycosidase Activities of 633 Strains of* Enterobacteriaceae and Vibrionaceae

	No. of strains examined	ONPG β-galactosidase	PNPG α-glucosidase	NPG β-glucosidase	PGUA β-glucuronidase	ONPX β-xylosidase
		Number of strains giving positive reactions				
Aeromonas (see Table 1)	18	18	(14)	14	0	0
Vibrio cholerae	11	11	(4)	(3)	0	0
Vibrio alginolyticus	10	0	0	0	0	0
Vibrio parahaemolyticus	1	0	0	0	0	0
Escherichia coli‡	115	106	(100)	(105)	109	1
Shigella dysenteriae 1	3	3	(3)	0	0	0
Shigella dysenteriae 2–10	10	0	(7)	(8)	7	0
Shigella flexneri	12	0	(12)	(6)	(3)	0
Shigella sonnei	14	14	(13)	(14)	14	0
Shigella boydii	5	0	(5)	(4)	1	0
Klebsiella pneumoniae	135	135	(135)	132	0	130
Klebsiella oxytoca	50	50	(48)	50	0	34
Klebsiella ozaenae	5	5	(5)	5	0	4
Klebsiella rhinoscleromatis	10	0	0	10	0	0
Enterobacter cloacae	36	36	(25)	28	0	34
Enterobacter aerogenes	29	29	(27)	27	0	27
Escherichia adecarboxylata	3	3	(2)	3	0	3
Erwinia herbicola	7	7	0	7	0	1
Yersinia pestis	1	1	(1)	1	0	1
Yersinia pseudotuberculosis	5	5	5	5	0	4
Yersinia enterocolitica	10	10	10	10	0	(4)
Serratia marcescens	14	14	(14)	14	0	0
Serratia liquefaciens	10	9	(10)	10	0	0
Serratia rubidaea	10	9	(9)	10	0	5
Hafnia alvei	10	9	(10)	(10)	0	0
Citrobacter (see Table 1)	17	17	(13)	(17)	0	0
Proteus (see Table 1)	67	0	0	0	0	0
Salmonella (see Table 1)	10	0	0	0	0	0
Edwardsiella tarda	6	0	0	0	0	0

Weak reactions are indicated by figures in brackets.
‡ Includes alkalescens-dispar strains.

nitrophenols released by the enzyme activity give a yellow colour only in alkaline solutions, the pH-values chosen were in all cases about 0.5 units higher than the pH-optima for the enzyme reactions as such.

The natural substrates for the glycosidases discussed in this paper are widely distributed in nature. The enzyme α-glucosidase catalyses the hydrolysis of a variety of α D-glucopyranosides including sucrose, maltose and turanose. β-glucosidase splits β 1 4 linked glucose oligosaccharides, cellobiose through cellohexaose as present in partial hydrolysate of cellulose. The enzyme β-glucuronidase which was found exclusively in strains of *Escherichia coli* and *Shigella* species, attacks all the na-

tural β-D-glucupyranosiduronates present for example in many polysaccharides. β xylosidase has the ability to split β-1-4 linked xylose oligosaccharides, from xylobiose to xylohexaose These substrates are present for example in partial hydrolysate of wheat flour (7) Finally various natural glycoproteins such as serumproteins, salivary glycoproteins, and glycoproteins of cellular membranes of which fucose is a component are potential substrates for α fucosidase.

Except for the β-glucosidase test, none of the present tests can be equated directly by conventional characterizing tests used in routine diagnostic bacteriology The β-glucosidase test is theoretically equivalent to the conventional test for cellobiose fermentation with the inherent possibility that discrepancies may arise owing to the different structure of the two substrates (3) A systematic comparison of the two tests was not included in the present study However if the results of the β-glucosidase test (NPG) listed in Table 3 are compared with the known pattern of cellobiose fermentation (6) a positive correlation between a strong reaction in the NPG test and the ability to ferment cellobiose will be revealed It is thus probable that the weak reactions seen in this test are of non-specific nature and that only strong reactions should be recorded as positive.

α-glucoside bonds are present in several compounds including the disaccharides maltose, sucrose and turanose. Thus, the α-glucosidase test demonstrates an enzyme possessing the ability to hydrolyse a number of different disaccharides used in conventional fermentation tests and thus, it cannot replace a single one of these tests. Differences in capacity to hydrolyse the chromogen and each of these potential substrates may arise from the presence or absence of specific permeases.

Because of reactions of intermediary colour intensity the two glucosidase tests presented problems in evaluating the results to be obtained if the tests were used in routine bacteriology Therefore, neither of these two tests is entirely satisfactory However the α-glucosidase test has been applied to *Haemophilus* taxonomy with some advantage (8)

None of the strains included in this study showed detectable α fucosidase activity This enzyme has previously been demonstrated in strains of *Clostridium perfringens* (1) in *Streptococcus mitior* (16) and in a gram-negative soil coccobacillus (14) Also certain strains of *Haemophilus* possess α-fucosidase activity as demonstrated by the present method (8)

As in *E coli* β-glucuronidase activity has previously also been found in certain types of *Streptococcus pyogenes* (18) in *S agalactiae* (17) in *S mitior* (15) in staphylococci, in certain corynebacteria and in sheep rumen microorganisms (13) It is noteworthy that, among all the species included in the present study only strains of *E. coli* and *Shigella* species possessed β-glucuronidase activity The high frequency of positive strains, especially of *E coli* (97 per cent) makes this test an extremely useful diagnostic tool and a possible means of rapid screening for these bacteria.

The β xylosidase activity of *Enterobacteriaceae* has previously been examined by *Brisou et al* (2) using a paper disc method. Their results are confirmed and somewhat extended by this study β-xylosidase activity was restricted to strains of the genera *Klebsiella*, *Enterobacter* and *Yersinia*. In addition, some strains of *Serratia rubidaea* possessed β xylosidase activity The frequency of positive strains in these genera was high. It is noteworthy that none of the strains of *K rhinoscleromatis* were ONPX positive.

The results obtained by the β-glucuronidase and β-xylosidase tests confirm the relationship between *Escherichia* and *Shigella* and between *Klebsiella* and *Enterobacter* It is suggested that *Escherichia adecarboxylata* being β-glucuronidase negative but β xylosidase positive is more closely related to the *Klebsiella Enterobacter* group than to *Escherichia* as proposed by *Leclerc* (10)

Using viable but non-multiplying suspensions of bacteria, the methods described in this paper are applicable to a large variety

of bacteria including organisms that do not grow in conventional test media (8). The methods are rapid and inexpensive. Furthermore, the quick result to be obtained by these methods has advantages in that sterile test substrates are not necessary.

We wish to thank Drs. *H. Bleeg*, *H. Lautrop* and *K. Gaarslev* for valuable suggestions.

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USE OF PREFORMED CAVITIES IN RABBITS FOR THE QUANTITATION OF LEUKOCYTE CHEMOTAXIS CAUSED BY BACTERIAL LIPOPOLYSACCHARIDES

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Sveen, K. & Hofstad, T. Use of preformed cavities in rabbits for the quantitation of leukocyte chemotaxis caused by bacterial lipopolysaccharides. Acta path. microbiol. scand. Sect. B, 84 252–258 1976.

Wound chambers implanted subcutaneously in rabbits proved suitable for measurements of leukocyte chemotaxis. Injection of bacterial lipopolysaccharide (LPS) in the chambers the sixth day after implantation was followed by a marked increase of polymorphonuclear leukocytes in the wound chamber fluid, the number of which was dependent on the time after application of LPS. Up to a certain amount of LPS the concentration of leukocytes in the chamber fluid was dose-dependent. The histopathological appearance of the granulation tissue lining the chamber wall one day after the injection of LPS from *Veillonella* revealed aggregation of blood cells plugging the lumina of small vessels and many eosinophilic leukocytes.

Key words: Rabbits; leukocyte chemotaxis; lipopolysaccharides.

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Until recently a reproducible method for measurement of leukocyte chemotaxis in experimental animals has not been available. The skin window method of *Rebuck & Crowley* (7) is easy to perform, but not accurate enough to be quantitative. In 1973 *Lundgren & Lindhe* (5) described a research model involving subcutaneous implantation of polytetrafluoroethylene (Teflon®) chambers in rats for analyzing chemotaxis in connection with newly formed granulation tissue and exudate. The present report describes the use of this method for the quantitation of the chemotactic activity of bacterial lipopolysaccharide (LPS) in rabbits.

MATERIALS AND METHODS

Source and Preparation of LPS

Packed wet cells of *Bacteroides fragilis ss. fragilis* strain Lille E 323 (8) and acetone-dried whole cells of *Veillonella parvula* strain Ve 9 (2) were extracted with 45 per cent aqueous phenol (11) at room temperature, and lipopolysaccharides (LPS) purified from the water phase by ultracentrifugation at 100,000 × *g* for one hour (3). The pellet was treated with ribonuclease and deoxyribonuclease, washed in distilled water and freeze-dried. While LPS-E 323 has a low endotoxic activity LPS-Ve 9 is a highly potent endotoxin, as measured by the local Shwartzman reaction and lethality in mice (manuscript in preparation). Before use, LPS was suspended in sterile isotonic saline, ultrasonicated (MSE-MULLARD 60 W 20 kc/s) for 2 min at 0 C, and, if necessary pH adjusted

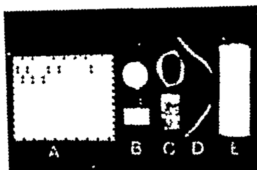


Fig. 1 The Teflon® chamber (E) and its different elements. The cylinder wall (A), the terminal cylinders (B), the keepers (C) and the small tube (D).



Fig. 2 Rabbit two days after implantation of ten chambers. Arrows indicate the medial part (top) of the chambers approximately 25 mm from the incisions.

to 7.2 with triethylamine. The very small amounts of triethylamine used had no chemotactic activity in preliminary experiments.

Chambers

The Teflon® chamber was 32 mm long and consisted of two 5 mm thick compact cylinders and a 0.4 mm thick cylinder wall (Fig. 1). The cylinder wall was perforated by holes of 1 mm diameter and the distance between two adjacent perforations was about 1 mm. At the end of the compact cylinders two holes were prepared and extended to the sides to fit the size of small tubes used to keep the cylinder wall tight to the compact cylinders. Keepers, consisting of 0.1 mm thick and 3 mm wide band were put terminally outside the cylinder wall. The internal volume of the chamber was about 800 µl.

Preparation of Rabbits and Implantation of Chambers

Six-month-old New Zealand white rabbits weighing 3.6-4.0 kg were used. Before implantation of the chambers the rabbits were clipped close to the skin on the dorsum and the lateral sides of the abdomen, and finally a hair remover (Nair® Charter Wallace Ltd., Kent, England) was used. The animals were anesthetized intramuscularly with Hypnorm Vet.® (Mekos AB, Helsingborg, Sweden) 0.5 ml/kg body weight. The shaved skin was then washed with 0.05 per cent Pyrnept® followed by 5 per cent iodine in ethanol. Working under aseptic conditions five incisions of 20 mm length and with a distance of 35 mm between each were made through the skin at the midline of the dorsum starting at a level corresponding to the forelegs. From these incisions lateral channels of 4.5 cm length were prepared between the subcutaneous muscle and the underlying fascia. Ten chambers, which had been sterilised in an autoclave for 30 min (134 °C), were then inserted into each rabbit. The incisions were closed with Teflex® (J. Pfrimmer & Co., Erlangen, Germany) 5-0 sutures and sprayed with Nobecutan® (Bofors Nobel-Pharma, Sweden) (Fig.)

Measurement of Chemotaxis

After disinfection of the skin with 5 per cent iodine in ethanol, the exudate in the chamber was aspirated through a perforation in the most descendant part of the chamber wall using a hypoderm needle and the volume measured. Twenty-five µl exudate were added to 475 µl of methylene blue and the number of leukocytes per µl of exudate was counted in a Bürker chamber. For calculation of the relative percentages of polymorphonuclear (PMN) and mononuclear (MN) leukocytes in the exudate (leukocyte score) smears were stained with May-Grunwald-Giemsa (Pappenheim staining).

Statistical Methods

Standard deviation was calculated as

$$\text{s.d.} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}} \quad (1)$$

The correlation between the corresponding doses and responses were tested according to the Pearson test (6).

Histological Examination

The chamber with adjacent tissue was removed from the anesthetized rabbit, and immediately fixed in 10 per cent phosphate buffered formalin. After dehydration, the blocks were embedded in paraffin cross sections were cut at 4 or 15 µm, and stained with hematoxylin-eosin.

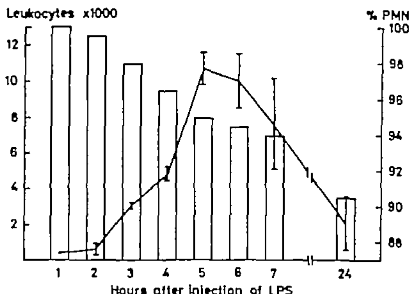


Fig 3 Numbers of leukocytes per µl of wound chamber fluid and the percentage of PMN at different times after application of 100 µg LPS-E 323 The time-response curv is drawn through the mean values of the leukocyte concentrations (3 rabbits) The vertical lines denote standard deviations.

EXPERIMENTS AND RESULTS

In the preliminary experiments, rabbits were sacrificed on different days after implantation of wound chambers. Five days after implantation the chambers were found to be incorporated in a grey mucous-like tissue, there was an ingrowth of granulation tissue through the perforations of the chamber wall. A continuous lining of the inside of the chamber wall with tissue was present six days after the implantation of the chambers. At this time, the chambers contained from 250–760 µl of exudate. The number of cells varied from 400 to 600 per µl, the leukocyte score of PMN and MN being calculated to 55 and 45 respectively

In order to examine the number of leukocytes and the percentage of PMN in the chambers at different times after injection of LPS the exudates were removed from the chambers in three rabbits six days after their implantation and replaced by 100 µg LPS-E 323 in 400 µl of sterile isotonic saline. The leukocyte score of the removed exudates was as indicated above. Every hour thereafter the wound chamber fluid was removed from one chamber in each rabbit and the cell content examined (Fig 3) The concentration of leukocytes became more than doubled between the fourth and fifth hour after application of LPS. Thereafter there was a steady decrease in cell number and an increasing amount of cells were disintegrated. One hour after injection of LPS all cells were polymorphonuclears, during the rest of the experimental period the proportion of PMN decreased slightly

The effect of different doses of LPS was tested by replacing the exudates in six chambers six days after implantation with the same number of two-fold dilutions of LPS in 400 µl of sterile isotonic saline. The exudates in the remaining four chambers (control chambers) were replaced by 400 µl sterile isotonic saline. In these and following experiments the test solutions were gently injected at a slight pressure. After five hours, the wound chamber fluid was removed from all chambers by aspiration. Both LPS preparations were used in this experiment. The chemotactic activity stimulated by different doses of LPS-Ve 9 and expressed as the con-

TABLE 1 *Numbers of Leukocytes per µl Wound Chamber Fluid of Three Rabbits 5 Hours after Injection of Different Concentrations of LPS V e 9 or Saline*

LPS (µg)	Rabbit A	Rabbit B	Rabbit C	Mean ± s.d.
50	12,800	13 700	15 700	14,066.6 ± 1 484.5
25	11,200	12,600	14,200	12,666.6 ± 1,501 1
12.5	9,600	11,000	12,000	10,866.6 ± 1,205.9
6.25	7,000	8,000	8,000	7,666.6 ± 577 4
3.12	5 400	6,200	3,600	5 066.6 ± 1,331.8
1.56	4 200	2,200	1,800	2,733.3 ± 1,285 4
Saline	460	520	560	526.6 ± 51.6
	420	540	600	

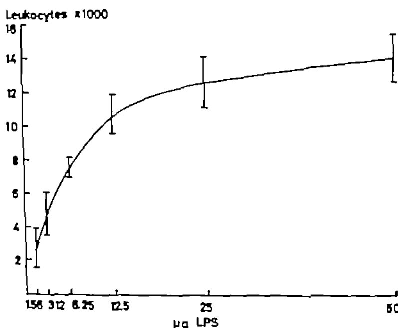


Fig. 4 Numbers of leukocytes per µl exudate following application of different doses of LPS-V 9 in the chambers. The dose-response curve is drawn through the mean values of the leukocyte concentrations (3 rabbits) The vertical lines denote standard deviations.

centration of leukocytes per µl wound chamber fluid, is shown in Table 1 No pronounced differences were found among the rabbits in their response to the different doses of LPS A positive correlation coefficient (r = 0.94) could be demonstrated in the ascending part of the dose response curve up to an amount of 12.5 µg of LPS-V e 9 (Fig 4) Exceeding that dose, the increase in leukocyte migration was insignificant. Analogous results were obtained with LPS-E 323 The dose-response curve was in principle the same but the ascending part of it was produced at a considerably higher range of LPS (6.25–100 µg) with a positive correlation (r = 0.97) for this part of the curve The control chambers harboured on an average 500 leukocytes per µl of wound fluid. The percentage of PMN and MN were 75 and 25 respectively while the LPS-induced fluid

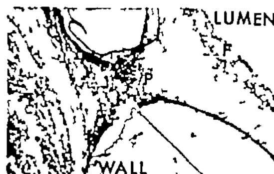


Fig 5 Cross section (4 µm) of a chamber wall (displaced) through a perforation (P) The day before removal and sectioning, the exudate in the chamber had been replaced by LPS-Ve 9 F = fibrin-like material. C = tissue-capsule (Magnification × 82.5)



Fig 6 Same chamber as in Fig 5 Cross section (15 µm) through the medially faced part of the chamber showing the vascularized tissue (VT) with vessel lumina (L) and the capsule (C) (detached from the chamber wall) (Magnification × 82.5)

TABLE 2. *Volume (µl) of Exudate the Wound Chambe s of Th e R bbits before and 5 Hou s after I jection of LPS V e 9 Saline*

Rabbit	Before injection		After injection of: LPS		Saline	
	Mean ± s.d.	Range	Mean ± s.d.	Range	Mean ± s d	Range
A	471 ± 104 1	340–600	247.5 ± 30 4	230–290	220 ± 15.3	205–235
B	349 ± 33 6	270–395	244 1 ± 90.5	150–395	172.5 ± 19 4	150–195
C	437 ± 59.3	305–595	285.8 ± 22.2	255–305	247.5 ± 11.9	235–260
A + B + C	419 ± 89 7	270–600	259 1 ± 55.6	150–395	213.3 ± 35.3	150–260

rated from the chambers contained 95 per PMN and 5 per cent MN The amount fluid in the chambers five hours after application of LPS was remarkably smaller than before application but larger than that of the control chambers injected with isotonic saline (Table 2) The chambers situated at the level of the hindlegs contained the smallest quantities of fluid.

Figs. 5 and 6 show cross sections through the wall of a chamber the exudate of which had been substituted for 5 hours by 25 µg of LPS-Ve 9 in 400 µl of saline the day before removal. Microscopic examination of the tissue in the lumen (Fig 5) revealed an amorphous fibrous-like mass rich in neutrophilic and eosinophilic leukocytes, but few mononuclear leukocytes and fibroblasts were found. At the site of the perforation of t hamber wall accumulations of neutroph served, but also here eosinophils predominated Young fibroblasts were seen close to the chamber wall. The fibrous capsule near the chamber wall was densely packed with fibroblasts, and the granulation tissue was extensively vascularized (Fig 6) The blood vessels were partly filled with cells (leukocytes and erythrocytes) the smaller vessels being completely packed. Neither accumulation of eosinophils and neutrophils in the granulation tissue, nor aggregation of cells in the small vessels, were found in sections from chambers injected with saline.

The chambers were well tolerated by all rabbits and no clinical signs of inflammation were observed up to three months after implantation. No microbial contamination of the was found six days after impl the chambers, or after injec tio ine.

DISCUSSION

The wound chamber method appears to be a suitable method for quantitative estimation of the chemotactic response of leukocytes to lipopolysaccharides. Several tests may be performed with the same test substance at the same time, or the chemotactic activity measured from different test substances, *e.g.* various LPS-preparations may be compared under equal biological conditions. Moreover knowledge about the biochemical aspects of chemotaxis can be obtained by chemical and biological examinations of the wound chamber fluid. Also, wound chambers may be usable in pharmacokinetic studies, for instance as an experimental model for determination of the concentration of antibiotics in exudates of inflammatory foci.

By using rabbits instead of rats, more chambers can be installed in the same animal, though this may be more expensive in terms of animals. When comparing the production of exudate in rabbits with that of rats, slightly more exudate was aspirated from the chamber six days after implantation in rabbits than after five days in rats (4). The exudate from the rats, however, harboured 2.6 times more leukocytes per µl.

No pronounced difference between the rabbits tested with respect to the total exudate aspirated from the chambers five hours after injection of LPS was found (cfr. Table 2). Neither was there any significant difference between the rabbits as to their responses to similar doses of LPS (cfr. Table 1). The finding of low leukocyte counts in the chambers filled with isotonic saline showed that no diffusion of test material from one chamber to another took place.

For up to 2 hours after application of LPS into the wound chambers there was no significant increase in the accumulation of leukocytes in the chamber fluid (cfr. Fig. 3). Up to certain amounts of LPS the attraction of polymorphonuclear leukocytes was dose dependent (cfr. Fig. 4).

Taken together the findings show that LPS induces a true positive chemotaxis of polymorphonuclear leukocytes, and that it most probably acts indirectly by inducing the formation of leukotactic mediators. This concept is in agreement with previous *in vitro* studies (9) and is supported by fractionation experiments which have shown that chemotactic agents of low molecular weight appear in the wound chamber fluid the first hour after application of LPS (unpublished results).

Aggregation of blood cells plugging the lumen of small vessels and a relatively high number of eosinophilic cells in the inflamed tissue are also found after preparing rabbits for the local Shwartzman reaction (10). Obviously bacterial LPS has a direct or indirect chemotactic effect on eosinophilic leukocytes. Whether this is part of a hypersensitivity reaction of the immediate type is not known.

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THE BACTERICIDAL ACTION OF BENZOIC ACID AND SODIUM ACETATE ON THE GRAM NEGATIVE FLORA OF DIALYSIS FLUID

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Kolmos, H. J. The bactericidal action of benzoic acid and sodium acetate on the gram-negative flora of dialysis fluid. Acta path. microbiol. scand. Sect. B, 84 259–264 1976.

Benzoic acid in concentrations from 0.18 per cent and upwards exerted a bactericidal action on the gram-negative flora of dialysis fluid at pH 4.75 *in vitro*. At a concentration of 0.32 per cent, a reduction of 10^{5} was obtained within 24 hours. Sulphonamide resistance did not affect the bactericidal action. The killing effect was hardly more than directly proportional to the concentration of benzoic acid. Increasing inoculum decreased the bactericidal effect. Therefore, benzoic acid should not be used as an artificial kidney disinfectant when the concentration of microorganisms exceeded 10^{3-4}/0.1 ml.

Key words: Benzoic acid; sodium acetate; bactericidal action; gram-negative flora.

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Within the last fifteen years, haemodialysis treatment of patients with chronic renal failure has become increasingly important. In order to reduce the costs of this treatment, efforts are made to re-use disposable dialysers, each patient re-using his own dialyser (8, 15). Because of microbial contamination of the dialysis fluid (5, 9) disinfection of dialysers to be re-used is essential. However, the use of toxic disinfectants like formaldehyde for this purpose may result in severe poisoning of the patients if the disinfectant is not sufficiently removed from the dialyser before re-use. In the search for less toxic agents, benzoic acid may be an alternative.

Benzoic acid is soluble in water to the extent of 0.37 per cent (4). The antimicrobial effect is due to the undissociated form (22) and the germicidal effect thus increases with a declining pH (6). P-aminobenzoic acid may inhibit its effect on some microorganisms (18). It is tolerated in oral doses of at least 12 g per day (16) and may be administered intravenously (1). It is conjugated in the liver and excreted in the urine as hippuric acid (17). Benzoic acid has a widespread use as a preservative (4, 7), before the antibiotic era it was used in the treatment of pyogenic infections (1). Reports on toxic effects in

man are sparse (10) but it may cause displacement of bilirubin from albumin *in vitro* (19)

The object of this study is to test the disinfecting effect of benzoic acid on microbially contaminated dialysis fluid.

MATERIAL AND METHODS

Specimens of dialysis fluid were taken from the canisters of recirculating single-pass monitors after 6 to 10 hours treatment and subsequently stored at 5 C for 6 to 10 hours before they were used. The dialysis fluid contained sodium, potassium, calcium, magnesium and chloride in concentrations equ valent to the extracell lar milieu sodium acetate nd nitrogenous waste products. It contained no glucose.

I order to obtain a pH of 4 75 the test solutions contained sodium acetate buffers. The germicidal effect was tested for the following concentrations

0.05% benzoic acid in 0.05 M acetate (5 expts.)
0.10% benzoic acid in 0 10 M acetat (5 expts.)
0 18% benzoic acid in 0 18 M acetate (9 expts.)
0.32% benzoic acid in 0.32 M acetate (12 expts.)

As controls served dialysis fluid in sodium acetate buffers of equivalent concentrations and dialysis *fluid in 0.9 per cent saline The test temperature* was 22 C.

For each concentration the number of surviving organisms were counted at the interv ls shown in Fig. 1 Colonies were counted on blood agar plates inoculated w th 0.1 ml from 10 fold series dilution in saline after 72 hours incubation at 22 C. Colonies from the controls in saline were identified at the start of each experiment, and the same applies to all test tubes at the end, using blood agar plates, Conradi-Drigalski plates, and Littmann plates. The media for identification were according to *Lautrop* (11) and *Møller* (14) All isolated strains were tested for their sensitiv ty to sulphonamides. Tablets containing sulphonamide (Rosco, Tåstrup, Denmark) were placed on inoculated 10 per cent blood agar plates without peptone which were incubated overnight at 35 C.

RESULTS

The microorganisms isolated from the dialysis fluid are shown in Table 1 *Citrobacter freundii* and *Klebsiella pneumoniae* were found in all samples. All strains of *Citrobacter freundii* were sensitive to sulphonamide (MIC < 6 μg/ml) while all strains of *Klebsiella pneumoniae* were resistant (MIC > 200 μg/ml) The identification of surviving microorganisms did not reveal a preponder ance of any single species at the three highest concentrations.

TABLE 1 *Microorganisms Isolated from 27 Specimens of D alysis Fluid*

Concentration/0.1 ml	Number of isolates				
	<10	10	10^5	10^6	Total
Citrobacte freu dii	1	2	7	17	27
Klebsiella pneumoniae	5	14	8	0	27
Pse domonas aeruginosa	5	1	2	0	8
Pseudomonas putida	2	3	1	0	6
Other Pseudomonad	3	4	0	0	7
Aeromonas formicans	1	2	0	0	3
Enterobacter cloacae	2	0	0	0	2
Yeasts	7	0	0	0	7

In all experiments log *N* (number of sur viving microorganisms) decreased with time. At 0 10 per cent benzoic acid the reaction velocity tended to decrease with time (all 5 experiments) At 0 18 per cent and 0.32 per cent benzoic acid it tended to increase (8 out of 9 and 10 out of 12 experiments, respectively) Representative inactivation curves are shown in Fig 1 Th mean velocities, k, determined by linear regression are shown in Table 2.

At 0.32 per cent, benzoic acid microorganisms could not be isolated from 0 1 ml after 24 hours. At 0 05 per cent, a primary linear fall was noted but growth occurred after 36 *to 48 hours (Fig 1) This was also true of* the 0 05 M acetate buffer alone where growth seemed to start a little earlier The growing microorganisms were yeasts.

The Wilcoxon non-parametric test (23) showed that the velocity constants obtained by the 0.32 per cent solutions were numerically higher than those obtained at 0 18 per cent ($2\alpha < 0.01$) The latter were numerically higher than the velocities obtained at 0.10 per cent ($2\alpha < 0.05$) The velocity constants at 0.10 per cent benzoic acid did not exceed those obtained by the 0 10 M acetate buffer alone ($2\alpha > 0.10$) The reaction velocity did

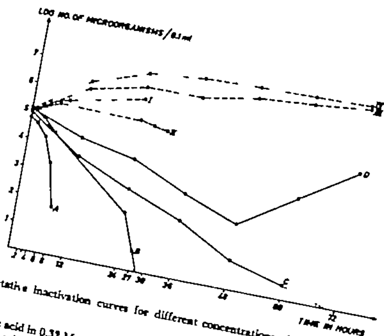


Fig. 1 Representative inactivation curves for different concentrations of benzoic acid in sodium acetate buffer
Signature
A: 0.32% benzoic acid in 0.32 M acetate
B: 0.18% benzoic acid in 0.18 M acetate
C: 0.10% benzoic acid in 0.10 M acetate
D: 0.05% benzoic acid in 0.05 M acetate
I–IV controls in saline.

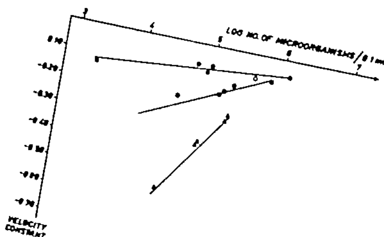


Fig. 2 The correlation between reaction velocity and size of inoculum at the three highest concentrations of benzoic acid.
Signature
● 0.10% benzoic acid in 0.10 M acetate
r = 0.897 y = 0.027 x 0.219
○ 0.18% benzoic acid in 0.18 M acetate
r = 0.939 y = 0.123 x 0.789
Δ 0.32% benzoic acid in 0.32 M acetate.
r = 0.821 y = 0.364 x 2.182

TABLE 2 *Velocity Constants Obtained at the Three Highest Concentrations of Benzoic Acid and Sodium Acetate Buffer*

	log N/0.1 ml		n	k_A	k_C
	start	end			
0.10% benzoic acid 0.10 M acetate	6.19	7.27	7	−0.059	−0.038
	4.97	7.00	6	−0.074	−0.075
	4.92	7.07	5	−0.099	−0.062
	4.76	7.03	5	−0.074	−0.071
	3.29	6.76	3	−0.137	−0.117
0.18% benzoic acid 0.18 M acetate	5.85	5.34	6	−0.086	−0.007
	5.60	6.09	6	−0.140	−0.014
	5.59	4.60	6	−0.083	0.000
	5.52	5.60	6	−0.124	−0.010
	5.20	5.34	6	−0.153	−0.041
	5.14	5.04	6	−0.164	−0.041
	4.72	5.71	5	−0.188	−0.021
	4.54	5.84	4	−0.199	−0.054
	4.04	4.18	3	−0.333	−0.060
0.32% benzoic acid 0.32 M acetate	5.30	5.68	6	−0.236	−0.006
	5.27	4.39	6	−0.258	−0.035
	5.21	5.52	6	−0.230	−0.001
	5.18	5.03	5	−0.471	−0.088
	5.16	5.31	5	−0.390	−0.055
	5.10	4.15	6	−0.237	−0.035
	4.94	4.94	5	−0.346	−0.068
	4.92	4.34	5	−0.361	−0.058
	4.77	5.21	5	−0.371	−0.022
	4.49	3.76	5	−0.506	−0.103
	4.43	5.29	4	−0.543	−0.040
	4.42	5.52	4	−0.706	−0.086

N: number of microorganisms in control in saline.
n: number of observations on inactivation curve.
k_A: velocity constant for benzoic acid in acetate buffer
k_C: velocity constant for acetate buffer

not increase with an increasing concentration of sodium acetate buffer (Table 2, k_C, $2\alpha >$ 0.10)

It applies to all three concentration sets of benzoic acid in acetate buffer that there was a positive correlation between the number of microorganisms present at start and the velocity constants (Table 2). This correlation seems to be linear within the range of inocula used (Fig 2). The slope of these lines increased with the concentration of benzoic acid.

The concentration exponent, n, calculated by means of the formula $(C_2/C_1)^n = k_2/k_1$ (21) will depend on the size of inoculum. If calculated from the two highest concentrations, C_2 = 0.32 per cent and C_1 = 0.18 per cent, at an inoculum size of $10^{5.0}$ (mean inoculum at the two highest concentrations) Fig 2 gives the corresponding velocities, k_2 = −0.358 and k_1 = −0.173 and n is thus 1.26.

DISCUSSION

The microbial contamination of the dialysis fluid used in this study corresponds to the results of previous studies (5, 9).

The bactericidal action of benzoic acid at the two lowest concentrations tested was doubtful and could be due to the *acetate*

buffer itself the lowest concentration favouring the growth of fungi which were occasionally present in small numbers in the dialysis fluid (Table 1) This is probably due to the low pH suppressing bacterial proliferation. At a concentration of 0.18 per cent benzoic acid, however a definite microbicidal effect was found and the effect increased with increasing concentration.

The results obtained in this study correspond to those obtained by *Lass* (12) who found that 0.15 per cent benzoic acid was effective against all bacteria. *Schmidt Lange* (20) showed that 0.15 to 0.18 per cent benzoic acid was necessary for the killing of *Staphylococcus aureus* However differences of culture media and pH make comparisons difficult.

For the description of the effect of disinfectants, *Madsen & Nyman* (13) could use the monomolecular reaction (log number of survivors rectilinear as a function of time, i.e. a constant velocity) *Eddy* (3) found increasing velocity as the number of surviving microorganisms diminished and, according to *Chick* (2) the monomolecular reaction was only applicable when the concentration of disinfectant was high or the concentration of microorganisms low The lack of conformity to the monomolecular reaction could also be ascribed to differences in sensitivity among the bacterial population exposed to the disinfectant.

In this study deviations from the monomolecular reaction were demonstrable at all concentrations tested. Although it could be shown that a second degree polynomium was a closer approximation, i.e. the variances of log N being smaller the monomolecular reaction was chosen as a more simple and yet reasonably close description allowing comparisons of velocities at different concentrations.

The lack of linearity may be ascribed to differences in sensitivity among the microorganisms since the dialysis fluid contained several species (Table 1) Such differences were only demonstrable at the lowest concentration, however It may also be due to the large inocula which corresponds to the

r*

correlation between the reaction velocity and the size of inoculum observed at the three highest concentrations (Fig 2) Whether this is due to the number of microorganisms *per se* or to inhibitors in the dialysis fluid in equivalent concentrations, however cannot be decided from these experiments.

The observation that p-aminobenzoic acid may inhibit the effect of benzoic acid (18) suggests a growth inhibitory mechanism similar to that of the sulphonamides. However this study revealed no differences in sensitivity of the sulphonamide resistant strains of *Klebsiella pneumoniae* and the sulphonamide sensitive strains of *Citrobacter freundii.*

The concentration exponent calculated from the two highest concentrations of benzoic acid indicates that the killing effect is hardly more than directly proportional to the concentration of benzoic acid.

The results of this study show that benzoic acid acts as a disinfectant on the gram negative flora in dialysis fluid *in vitro* At 0.32 per cent, a reduction by 10^{4} is reached within 24 hours. The observed effect of inoculum size implies that it should not be used as an artificial kidney disinfectant when the concentration of microorganisms exceeds 10^{3-4}/0 1 ml. The latter need not be a problem since the disinfection is always preceded by a mechanical rinsing in order to remove remnants of blood from the dialyser In addition dialysis fluid is seldom microbially contaminated to the same degree as in this study

I am grateful to *M Weis Bentzon* actuary Biostatistical Department, Statens Seruminstitut, for his help with the statistical analyses.

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ACQUIRED RESISTANCE OF BCG VACCINATED RED MICE TO INFECTION WITH *LISTERIA MONOCYTOGENES*

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Jespersen, A. Acquired resistance of BCG-vaccinated red mice to infection with *Listeria monocytogenes*. Acta path. microbiol. scand. Sect. B, 84 265–272, 1976.

Infection experiments have shown that red mice, belonging to the vole family could be infected with *Listeria monocytogenes* and that they were almost as susceptible to intravenous infection as CF mice. Vaccination of red mice with BCG induced a resistance which could be demonstrated by prolongation of the survival time of the animals after challenge with Listeria. The resistance was greatest in the second and third week after vaccination and was considerably higher after intravenous injection of BCG vaccine than after intraperitoneal or subcutaneous injection. On the basis of studies concerning the dose of vaccine, the route of vaccination, and the interval between vaccination and challenge, a method by which to evaluate the potency of a strain of BCG has been elaborated By this method it was found that there was a significant difference between the resistance produced by a strain of BCG which is weakly virulent and one which is strongly virulent for hamsters.

Key words *Listeria monocytogenes* red mice native resistance BCG-induced resistance potency of BCG strains.

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Various species of mice belonging to the murid family have been used in the course of time in infection experiments with *Listeria monocytogenes* (*L.m.*) whereas the susceptibility of voles to *L.m.* has not been examined. Red mice like rabbits, are strongly resistant to *M tuberculosis* but very sensitive to *M bovis* (*Jespersen* 1954) Vaccination of red mice with BCG vaccine induces a resistance to infection with *M bovis* the degree of which is dependent on the BCG strain used (*Jespersen & Bentzon* 1964 a, b) Since rabbits are sensitive to *L.m.*, the same might also apply to red mice.

The aim of the present experiment was to examine whether red mice can be infected with *L.m.* and whether the resistance induced by BCG is sufficiently high to enable the development of a method for determination of the potency of a strain of BCG

MATERIALS AND METHODS

Animals The red mice and the CF mice were bred at the farm belonging to Statens Seruminstitut. The animals, 2–5 months old at the commencement of the experiment, were distributed at random into earthenware jars, one animal in each jar The individual experimental groups consisted of equal numbers of female and male animals.

BCG-vaccine I expts. 3 and 4 a freeze-dried

TABLE 1. *Survival Times (in Days) of Groups of Red Mice Injected Intravenously with Varying Doses of* L.m.

10^{-3} 0.1 ml i.v.	0.33×10^{-2} 0.1 ml i.v.	10^{-2} 0.1 ml i.v.	0.33×10^{-1} 0.1 ml i.v.	10^{-1} 0.1 ml i.v.
4	5	3	3	2
6	5	3	3	2
s	5	3	3	2
s	6	3	3	2
s	9	4	3	3
s	s	4	3	3
s	s	4	5	3
s	s	6	7	3

vaccine produced at the BCG Department, Statens Seruminstitut, was used. The strains of BCG used in expt. 5 were grown in Dubos fluid Tween medium and dispersed by ultrasonics. The strain Copenhagen was received from the BCG Department, Statens Seruminstitut, as a freeze-dried routine culture, batch 60 produced on March 17, 1971. This strain is considerably more virulent for hamsters than the Prague strain, but not as virulent as the strongest BCG strains. The strain Prague, received as a freeze-dried culture, batch 725, was prepared from the Prague strain on May 12, 1969 by the BCG Department, Statens Seruminstitut. This strain is weakly virulent for hamsters. Each of the two strains was subcultured twice in Dubos medium, after which the culture was treated with ultrasonics for ten minutes (*Jespersen & Bentzon* 1964 a). The number of viable units in the doses used for vaccination was determined by inoculation of suitable dilutions on Löwenstein-Jensen medium.

Listeria monocytogenes. The strain (SSC 1423) provided by Dr. *Jørgen Bennedsen* has been described previously, including details concerning its virulence for C3H mice and its maintenance (*Bennedsen & Olesen Larsen* 1975). A suspension of *L.m.* in broth was adjusted to a density of 0.025 in Coleman electrophotometer (10^{9}) and tenfold dilutions down to 10^{-7} were made with redistilled water. Two blood agar plates were inoculated with five drops of 0.05 ml per plate of each of the dilutions 10^{-5}, 10^{-6} and 10^{-7}.

Vaccination. Subcutaneous vaccination was carried out by injection of the vaccine into the right groin and intravenous vaccination by injection into a tail vein.

Challenge. Challenge was made by injection into a tail vein of 0.1 ml of a standard suspension diluted 100 times (10^{-2}). This dose corresponds to about 2 × 10 viable organisms. The animals were challenged at random.

Measurement of resistance. Survival times after challenge were used for determination of the resistance. The mice were observed for ten days after the challenge injection.

Statistical methods: Evaluation of the results was based on the combination of the number of surviving animals and the survival times of animals which died spontaneously. In the infection experiments, LD_{50} and the slope of the log dose-response curve were determined by the logit

TABLE 2. *Survival Times (in Days) of Groups of Red Mice and CF Mice Injected Intravenously with Varying Doses of* L.m.

0.25×10^{-2} 0.1 ml i.v.	0.5×10^{-2} 0.1 ml i.v.	10^{-2} 0.1 ml i.v.	10^{-2} 0.2 ml i.v.
Red mice			
5	6	4	2
5	7	4	3
s	8	4	3
s	8	4	3
s	s	4	4
		6	4
		6	4
		6	4
s	s	s	5
s	s	s	5
CF mice			
3	3	3	2
4	3	3	2
4	3	3	2
4	4	3	2
4	5	4	2
7	s	s	2
		s	3
	s		3
s	s	s	8
			s

method on the basis of the number of surviving animals (Finney 1964) Comparison of the survival times was performed by Wilcoxon's rank sum test (*Wilcoxon* 1945)

RESULTS

Expt. 1 Natural Resistance of Red Mice to Listeria

Table 1 shows the survival times, in days, of red mice injected with varying doses of *L.m.* The three largest doses killed all the animals in the group and the survival times were prolonged the smaller the dose. Five out of eight animals died in the group injected with the next smallest dose, and two out of eight in the group injected with the smallest dose.

Statistical analysis The course of the infection was clearly dose-dependent. The slope of the dose response curve was 4.59 (1.31–7.87) LD_{50} 0.20×10^{-4}

Expt. 2 Comparison of the Natural Resistance of Red Mice and CF Mice to Listeria

Table 2 shows the survival times, in days, of groups of red mice and CF mice injected intravenously with varying doses of *L.m.* The susceptibility of the two animal species to Listeria cannot be compared on the basis of LD_{50} since this value cannot be determined in the case of CF mice (see below) If the susceptibility is evaluated on the basis of the

TABLE 3 *Survival Times (in Days) of Groups of Red Mice Vaccinated Subcutaneously with 1.2 × 10⁸ Viable Units of Freeze-dried BCG Vaccine and Challenged Intravenously with 0.1 ml 10^{-4} L.m 1 2 3 and 4 Weeks Later*

Interval between vaccination and challenge 1 week	2 weeks	3 weeks	4 weeks
3	4	5	4
3	5	9	4
4		[illegible]	6
4	[illegible]	[illegible]	7
6			8
	[illegible]		10

lowest dose that can kill all the mice in a group the dose is 0.2 ml 10^{-2} for both species, but the survival times of the CF_1 mice are shorter than those of the red mice. This latter finding applies irrespective of the dosage.

Statistical analysis As regards the red mice, the slope of the dose-response curve was 5.52 (2.20–8.84) i.e. similar to that found in Expt. 1 In the case of the CF_1 mice, the slope was 1.32 (−0.7–3.34) Since the slope is not significantly different from 0 LD_{50} cannot be determined

Expt 3 BCG-induced Resistance—Time of Occurrence of Maximum Degree of Resistance

In order to examine whether BCG vaccine can produce resistance against Listeria infection, and to ascertain the time at which resistance is at a maximum, groups of red mice were vaccinated subcutaneously with 0.03 mg (1.2×10^{8} viable units) of freeze-dried BCG and challenged intravenously with 0.1 ml 10^{-4} *L.m* one, two, three and four weeks after vaccination. Table 3 shows the survival times, in days, of the four groups of mice. One week after vaccination, the survival times were the same as those in the corresponding group of non vaccinated mice in Expts. 1 4 and 5 After two and three weeks, a considerable resistance had developed. In both groups, four out of six mice survived and the survival times of the survivors were prolonged. Four weeks after vaccination, the resistance had decreased though it was greater than after the first week.

Statistical analysis Changes in the distribution of the survival times during intervals from the first to the second week and from the third to the fourth week, were in both cases near the 5 per cent significance limit (rank sum 1st week = 28 expected 39 p = 5–10 per cent. Rank sum 4th week = 26, expected 39 p = 2–5 per cent) Thus, it is justifiable to conclude that the resistance of the animals was greatest in the second to third week.

TABLE 4 *Survival Times (in Days) of Non-vaccinated Red Mice and Mice Vaccinated Subcutaneously Intraperitoneally or Intravenously with Graded Doses (Viable Units) of Freeze-dried BCG Vaccine Challenged Intravenously with 0.1 ml* $10^{-[illegible]}$ L.m. *Three Weeks after Vaccination*

Non-vaccinated	Subcutaneous vaccination				Intraperitoneal vaccination				Intravenous vaccination			
	2.1×10^2	2.1×10^4	2.1×10^6	2.1×10^8	2.1×10^2	2.1×10^4	2.1×10^6	2.1×10^8	2.1×10^2	2.1×10^4	2.1×10^6	2.1×10^8
3	3	4	4	5	4	4	5	4	4	4	10	7
3	4	4	4	6	4	4	7	7	4	6	s	s
4	4	6	5	7	4	4	8	7	5	s	s	s
4	5	7	5	7	4	4	8	8	5	s	s	s
4	5	7	5	9	5	5	9	s	5	s	s	
4	6	8	6	s	5	8	s	s	8	s	s	s
4	s	s	7	s	5	s	s	s	s	s	s	s
5	s	s	8	s	6	s	s	s	s	s	s	s
7	s	s	9	s	s	s	s	s	s	s	s	s
	s	s	s	s	s	s	s	‡	s	s	s	s

s Survivor
‡ Died during the interval between vaccination and challenge.
Underlined figures = Median survival times.

Expt. 4 Effect of the Route of Vaccination on BCG-induced Resistance

Table 4 shows the survival times, in days, of groups of mice vaccinated subcutaneously intraperitoneally or intravenously with graded doses of BCG vaccine. Together with a non-vaccinated group they were challenged intravenously with *L.m.* three weeks after vaccination. All non-vaccinated mice except one died. The median survival time, expressed as the survival times of the two middle animals in the group, (underlined in the Table) was four days. Among the vaccinated mice, the highest resistance was found in animals injected intravenously. This was particularly evident in the groups given the three highest doses, where the number of survivors after increasing doses was eight, nine and nine. In the group vaccinated with 2.1×10^3 viable units, the median survival time was 6.5 days, and four animals survived as against one in the non-vaccinated group. The resistance was slightly increased in all groups of animals vaccinated subcutaneously and intraperitoneally and there was no difference in the resistance after subcutaneous and intraperitoneal injection, whether estimated on the basis of median survival times or on the number of surviving animals.

Statistical analysis. No difference was found in the effect of the three largest doses regardless of the vaccination route. The total frequency of surviving animals injected with these doses was 10/30, 14/29 and 26/30 after subcutaneous, intraperitoneal and intravenous vaccination, respectively. The frequency after intravenous vaccination was significantly higher than that after intraperitoneal and subcutaneous vaccination ($p = 0.3$ per cent

TABLE 5. *Survival Times (in Days) of Non-vaccinated Red Mice and Mice Vaccinated Intravenously with Graded Doses (Viable Units) of Living BCG Vaccine (Strains Prague and Copenhagen) and a Heat-killed Copenhagen Strain and Challenged Intravenously with 0.1 ml 10^{-3} L.m. Three Weeks after Vaccination*

Non-vaccinated	Prague (living) BCG 2.5×10^3	Prague (living) BCG 2.5×10^4	Prague (living) BCG 2.5×10^6	Copenhagen (living) BCG 1.5×10^2	Copenhagen (living) BCG 1.5×10^4	Copenhagen (living) BCG 1.5×10^6	Copenhagen (killed) BCG 1.5×10^3	Copenhagen (killed) BCG 1.5×10^4	Copenhagen (killed) BCG 1.5×10^6
3	3	4	3	3	3	4	3	3	3
3	3	4	3	4	5	5	3	3	3
3	4	4	4	4	5	5	3	4	4
3	4	4	4	4	5	6	3	4	4
3	4	4	4	5	5	6	3	4	4
4	4	4	4	5	6	6	3	4	4
4	4	4	4	6	s	7	4	4	4
4	4	4	4	9	s	7	4	4	4
4	4	4	5	9	s	9	4	4	4
4	4	5	5	9	s	9	4	4	4
5	4	5	5	s	s	s	4	4	4
5	5	5	5		s	s	4	5	5
5	5	5			s		4	5	5
5	5	5	s		s		5	5	5
5	6	10			s		5	5	6
5	6				s		5	5	6
6	9				s		6	5	6
6		s			s		10	7	7
s		s			s		s		s
s		s	‡		‡	s	s		

s Survivor

‡ Died during the interval between vaccination and challenge.

Underlined figures = Median survival times.

and 0.01 per cent, respectively) with the last two routes, the mutual difference in frequency was not significant ($p > 10$ per cent)

Expt 5 BCG Resistance Induced by Strains of Varying Virulence

Using a method based on the results of the above-mentioned experiments, comparison was made of the effect of two viable vaccines produced from a strain Prague, which is weakly virulent and another strain, Copenhagen, which is strongly virulent for hamsters. The latter vaccine was also examined in heat killed form. Dilutions 10^{-1} 10^{-2} and 10^{-3} of the two strains cultured in Dubos fluid medium and the same dilutions of the Copenhagen strain killed by heat were injected intravenously into groups of 20 mice. Three weeks after vaccination, these animals and a non-vaccinated group were challenged with 0 1 ml 10^{-} *L.m* injected intravenously The survival times after challenge of the animals in the ten groups are shown in Table 5 In the non vaccinated control group, all animals except two died, and the survival times of the two middle animals in the group were 4 and 5 days. In the groups vaccinated with killed BCG vaccine, there was no increased resistance neither the number of survivors nor the survival times in any of the groups deviated from those of the control animals. The two highest doses of the Prague vaccine induced significant resistance. The number of surviving animals was 5 and 7 respectively as against 2 in the control group. On the other hand, the median survival times were not prolonged. The group vaccinated with the lowest dose did not differ from the control group All doses of the Copenhagen vaccine induced considerable resistance, whether estimated on the basis of the number of surviving animals, viz. 10, 13 and 10 with the three doses, or on the basis of the median survival times.

Statistical analysis Irrespective of the vaccines used, the effect of dosage was not significant. The frequencies of survival of animals vaccinated with Copenhagen, Prague and heat killed vaccine were 33/59 15/59 and 6/60 respectively Using the Copenhagen vaccine, the frequency was significantly higher than that observed after the other two vaccines ($p = 0\ 15$ per cent and $p < 0.01$ per cent) and the frequency obtained with the Prague vaccine was significantly higher than that obtained with the heat killed vaccine ($p = 3\ 6$ per cent)

On the 69th day after the first challenge, the surviving animals in all groups were injected intravenously with 0.2 ml 10^{-1} *L.m* This dose was only able to kill four animals, all belonging to the Copenhagen group. Twenty-eight days after the second challenge, the survivors were injected with 0.5 ml 10^{-} *L.m* This time three animals, also belonging in the Copenhagen group, died.

In a pilot study in which the same vaccines were used but where the doses of the viable vaccines were ten times lower the surviving animals were injected intravenously with 0.2 ml 10^{-} *L.m.* 84 days after the first challenge. Once more the number of deaths was highest in the Copenhagen group where 29 out of a total of 69 animals died (42 per cent) Among the animals vaccinated with the Prague vaccine only 18 per cent died (7 out of 38) and among those vaccinated with heat killed vaccine only 15 per cent died (4 out of 27)

DISCUSSION

The natural resistance to *Listeria monocytogenes* in mice of the murid family seems to be different in the individual strains of mice. Thus, *Collins & Mackaness* (1970) found that LD_{50} for CD mice was 5×10^{3} viable organisms injected intravenously while *Mackaness* (1962) and *Ackerman* (1964) using *Hall* mice, reported doses of 1×10^{6} and 3×10^{3} respectively The same strain of Listeria was used in the experiments.

As in the case of the murids, red mice could be infected with *L.m.* Comparison of the natural resistance in red mice and CF mice showed that the lowest dose of Listeria which killed all the mice in a group was the same for both species of mice, viz. 0.2 ml

10 corresponding to about 10^{7} viable organisms. LD_{50} for red mice was about one quarter of that dose. LD_{50} for CF_1 mice can not be stated since the slope of the dose-reaction curve in the area covered by the experiment did not differ significantly from 0.

Vaccination of red mice with BCG induced considerable resistance against infection with Listeria. After subcutaneous injection of 0.05 mg freeze-dried vaccine, the resistance was greatest two to three weeks after the vaccination. Subsequently it decreased, though it was still demonstrable after four weeks. Intravenous injection caused a considerably higher degree of resistance than subcutaneous and intraperitoneal injections, both of which had an equally weak effect.

The main aim of the present work was to examine whether the resistance to a Listeria infection in BCG vaccinated red mice determined by the survival times after challenge could be used to evaluate the potency of a BCG strain. Two strains of BCG were used, Prague which is weakly virulent for hamsters and Copenhagen which is strongly virulent. Using vaccine doses of 0.2 ml of dilutions 10 10^{-4} and 10^{-} of an ultrasonic treated Dubos culture, an interval of three weeks between vaccination and challenge, and a challenge dose of 0.1 ml 10^{-2} a significant difference in the protective capacity of the Prague and Copenhagen strains was found.

The difference in the inhibitive effect of the two BCG strains on the multiplication of Listeria also became apparent when the mice which survived the first challenge were infected with a new and larger dose of Listeria. Even though the dose was twenty times greater than that used for the first challenge, it was still too small since only four animals died. A third challenge with an even larger dose resulted in the death of a further three animals, but all seven animals were vaccinated with the Copenhagen strain. In a preliminary study where the mice were vaccinated with the same strains but with ten times lower doses, 42 per cent of the animals vaccinated with the Copenhagen strain died after the second challenge as against 18 per cent of those vaccinated with the Prague strain and 15 per cent of those vaccinated with the heat killed vaccine. The explanation must be that the vaccine produced from the potent BCG strain had a strongly inhibitory effect on the multiplication of Listeria, thus resulting only in a weak specific resistance. On the other hand, the killed vaccine and the vaccine produced from the weak BCG strain were able to inhibit the multiplication of Listeria to a much smaller extent, therefore resulting in the development of a strong specific resistance.

The low potency of the Prague strain has been demonstrated in several animal experiments. This applies to its immunogenic potency against tuberculous infection in guinea pigs (*Sula* 1963) its virulence for guinea pigs and hamsters (*Jensen & Kjær* 1957 *Bunch-Christensen et al.* 1968) and its allergenic potency measured in guinea pigs and hamsters (*BCG Department Statens Serum institut Copenhagen* 1965 *Jespersen* 1973). Its weak potency has also been observed in vaccination of human beings. A comparison on children of vaccines prepared at various production centres showed that the Prague strain gave the smallest tuberculin reactions and the smallest vaccination lesions (*Nyboe & Bunch-Christensen* 1966).

The Copenhagen strain is considerably more virulent for hamsters than the Prague strain, though it is not as virulent as the strongest BCG strains.

Experiments using the two BCG strains for vaccination of red mice have shown that the resistance to Listeria infection is stronger the more virulent the BCG strain. The same applies to the resistance of red mice and guinea pigs to infection with virulent tubercle bacilli (*Jespersen & Bentzon* 1964 a, b, *Jespersen & Bentzon* 1967). Thus a correlation has been found between the protective effect of a BCG vaccine against Listeria infection and its protective effect against virulent tubercle bacilli, and consequently also its protective effect against tuberculous infection in human beings (*Jespersen* 1971).

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TUBERCULIN SHOCK IN RED MICE AND CF1 MICE IMMUNIZED WITH STRAINS OF BCG OR *MYCOBACTERIUM TUBERCULOSIS*

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Jespersen, A. Tuberculin shock in red mice and CF1 mice immunized with strains of BCG or *Mycobacterium tuberculosis*. Acta path. microbiol. scand. Sect. B, 84 275–279 1976.

Groups of red mice and CF1 mice immunized intravenously with varying doses of a weak and a strong strain of BCG and a strain of *M. tuberculosis* were challenged 3–4 weeks later with 2 or 0.5 mg of purified tuberculin injected intravenously. The shock sensitivity of the animals in the individual groups was evaluated on the basis of the number of deaths and the survival times after challenge. In the red mice, the strain of *M. tuberculosis* induced a significantly greater sensitivity than the BCG strains. The strong strain of BCG induced a slightly greater sensitivity than the weak strain, but the difference was not significant. The CF1 mice were more sensitive to tuberculin shock than the red mice, but any difference in the sensitivity of the animals in the individual groups immunized with the three strains could not be demonstrated.

Key words: Tuberculin shock; red mice; CF1 mice; BCG-induced sensitivity; *Mycobacterium tuberculosis*-induced sensitivity; potency of mycobacterial strains.

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In vivo manifestations of delayed type hypersensitivity in tuberculous mice or in mice immunized with BCG have been demonstrated only during the last two to three decades.

Various strains of the murid family have been used as experimental animals, but red mice or other members of the vole family have not been examined. In the present study the shock sensitivity of red mice and CF1 mice immunized with BCG or *M. tuberculosis* has been compared.

MATERIALS AND METHODS

Animals. In this study and the simultaneously performed *Listeria* immunity experiments (*Jespersen* 1976) use was made of red mice and CF1 mice bred at the farm attached to Statens Seruminstitut. The animals, which were 4–7 months old at the commencement of the study, were distributed at random into earthenware jars with one animal in each. The various experimental groups consisted of equal numbers of females and males. Hamsters aged 4–7 months bred at the same place as the mice were used for the determination of the virulence of the BCG strains (*Jespersen* 1964, *Jespersen & Bentzon* 1964).

Mycobacterial strains. The BCG strains were used in a previous experiment (*Jespersen* 1973) where details of their origin are given. The Prague strain was weakly virulent for hamsters and the Copenhagen strain was considerably stronger. The strain of *M. tuberculosis* (E 36499) was isolated from a patient in September 1971 and maintained by culture and passages in animals, the last being injection of the strain into guinea pig in July 1975.

Production of bacterial suspensions for immuni-

zation: Löwenstein-Jensen cultures of the three strains were grown in Dubos fluid medium with Tween and subcultured twice before injection of the animals. Three-quarters of the supernatant fluid was drawn from a flask of undiluted culture before the sediment was shaken. This suspension was diluted with equal parts of diluted Sauton (one part medium to three parts distilled water). This second suspension was again diluted in the same way. The three suspensions were used for immunization of the animals, each of which was given 0.5 ml.

Inoculation of suitable dilutions of the suspension of BCG Prague on to Löwenstein-Jensen medium showed that the number of viable units in the lowest immunization dose was 1.9×10^7; the corresponding number applying to BCG Copenhagen was 3.3×10^7.

The culture of *M. tuberculosis* was treated with ultrasonics. Doses of 0.2 ml of an undiluted culture, of a tenfold diluted culture and of a hundredfold diluted culture were used for immunization in Expt. 1. The lowest dose contained 14×10^5 viable units. The doses used in Expt. 2 were half as large as those in Expt. 1. All the mice (red mice and CF1 mice) were immunized at random.

Virulence determination of BCG strains on hamsters. Doses of 3.8×10^7 viable units of BCG Prague and 6.5×10 viable units of BCG Copenhagen were injected intraperitoneally into 20 hamsters. The number of animals which died spontaneously was recorded for 180 days after the injection at which time the survivors were killed. BCG Copenhagen was clearly the most virulent of the two strains. Among the 20 animals in the group 15 died (mean survival time 136 days). In contrast, none of the 20 animals injected with BCG Prague died.

*Tuberculin**. Challenge was carried out with purified tuberculin PPD RT 33. 10 mg/ml in Expt. 1 (2 mg per mouse) and 2.5 mg/ml in Expt. 2 (0.5 mg per mouse).

Experimental

Expt. 1. Groups of 10 red mice and 10 CF1 mice were immunized intravenously with the above-mentioned three doses of BCG Prague, BCG Copenhagen and *M. tuberculosis*. Three weeks later these mice, together with a non-immunized control group were challenged intravenously with 2 mg purified tuberculin.

Expt. 2. Corresponding groups of red mice and CF1 mice were immunized with the same doses of BCG as in Expt. 1 and with half doses of *M. tuberculosis*. Since a dose of 2 mg tuberculin had proved to be too big for the CF1 mice, the challenge dose given 4 weeks after immunization was reduced to 0.5 mg purified tuberculin.

In both experiments, the number of animals which died spontaneously was recorded daily up to and including the 7th day after challenge. Observation of the animals was concluded after 10 days.

Statistical analysis: Comparison of the various groups was made by means of a modification of the *Wilcoxon* (1945) rank-sum test compiled by *Larsen* (to be published). The survival times are divided into five groups: 1st, 2nd, 3rd, 4th–7th, and >7th days. P values less than 5 per cent are considered significant and are shown in brackets in the text.

RESULTS

Experiment 1

Table 1 shows the survival times of red mice and CF1 mice immunized intravenously with varying doses of BCG Prague, BCG Copenhagen and *M. tuberculosis* and challenged 3 weeks later intravenously with 2 mg PPD (100 000 TU).

Red mice. All the animals in the non-immunized control group survived. Within the individual groups immunized with the BCG strains or the strain of *M. tuberculosis*, there was a slight reduction in the survival times the higher the dose. This is most evident if BCG Prague is used and least evident if *M. tuberculosis* is used. Comparison of the groups given similar numbers of viable units, i.e. those immunized with 14×10^6 v.u. *M. tuberculosis*, 3.3×10^8 v.u. BCG Copenhagen and 3.8×10^7 v.u. BCG Prague, shows that the survival times are shortest in the first group, longest in the third and intermediate in the second group.

Statistical analysis. The difference in survival times of animals immunized with 14×10^6 *M. tuberculosis* and of those immunized with 3.8×10 BCG Prague is significant (<0.1 per cent). The difference between the first group and that immunized with 3.3×10^8 BCG Copenhagen is also significant (0.6 per cent). However, the difference between the groups immunized with 3.3×10 BCG Copenhagen and 3.8×10^7 BCG Prague is not significant. Nor is there any difference between the groups immunized with 6.5×10^8

* The writer is grateful to *Mogens Magnusson*, Chief of the Tuberculin Department, for the preparation and provision of the tuberculin.

TABLE 1 *Survival Times in Days of Red Mice and CF1 Mice Immunized Intravenously with Various Doses (Viable Units) of BCG Prague BCG Copenhagen and* M. tuberculosis *Strains and Together with Non-immunized Control Group Challenged Intravenously 3 Weeks Later with 2 mg Purified Tuberculin 100,000 TU*

Non-immunized controls	BCG Prague 1.9×10^7	BCG Prague 3.8×10^7	BCG Prague 7.6×10^7	BCG Copenhagen 3.3×10^7	BCG Copenhagen 6.5×10^7	BCG Copenhagen 13×10^7	*M tuberculosis* 14×10^6	*M tuberculosis* 14×10^6	*M tuberculosis* 14×10^7
Red mice									
s	2	2	1	2	1	1	1	1	1
s	5	2	2	2	1	2	1	1	1
s	5	3	2	2	2	2	1	1	1
s	6	3	2	2	2	2	1	1	1
s	6	4	2	3	2	2	2	1	1
s	s	5	2	3		2	2	1	2
s	s	5	2	3	2	3	2	2	2
s	s	5	2	3	2	3	3	2	2
s	s	s	3	3	3	5	6	2	2
s	s	‡	7	s	4	‡	6	‡	‡
CF1 mice									
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	1	1	1	1	1	1	1	1	1
s	s	1	1	1	1	1	1	1	1
s	s	s	‡	s		s	s	1	1

s = Survivor
‡ = Died in the interval between immunization and challenge.

BCG Copenhagen and 7.6×10^7 BCG Prague.

CF1 mice With the exception of a few animals, all the immunized CF1 mice died within the first 24 hours. On the other hand, none of the non-immunized control animals died.

Experiment 2

In this experiment, both the red mice and the CF1 mice were immunized with the same doses of BCG Prague and BCG Copenhagen, and with doses of *M tuberculosis* which were half as large as those used in Expt. 1 After 4 weeks, these animals and a non-immunized control group were challenged intravenously with 0.5 mg PPD (25,000 TU) since the dose of 2 mg PPD used in Expt. 1 had proved to be too high for the CF1 mice The survival times after challenge are shown in Table 2.

Red mice There is a slight dose dependence in the groups immunized with the BCG strains, with a tendency to decreasing survival times with increasing doses. On the other hand, in the group immunized with *M tuberculosis* the sensitivity decreases with increasing doses. This is probably because *M tuberculosis* has a greater virulence for red mice than BCG Thus, in the group immunized with the highest dose of *M tuberculosis* four mice died in the period between immunization and challenge, as against three and one in the corresponding groups immunized with BCG Copenhagen and BCG Prague respectively Comparison of the groups immunized with similar doses shows that the survival

TABLE 2 *Survival Times in Days of Red Mice and CF1 Mice Immunized Intravenously with Various Doses (Viable Units) of BCG Prague BCG Copenhagen and M. tuberculosis Strains and Together with a Non-immunized Control Group Challenged Intravenously 4 Weeks Later with 0.5 mg Purified Tuberculin 25,000 TU*

Non-immunized controls	BCG Prague			BCG Copenhagen			*M tuberculosis*		
	1.9×10^7	3.8×10	7.6×10^7	3.3×10^7	6.5×10	13×10^7	7×10^6	7×10^6	7×10^7
Red mice									
*	1	3	2	2	2	2	1	1	2
*	*	3	3	2	2	2	1	2	2
*	*	4	4	3	2	2	1	2	3
*	*	4	4	3	3	2	2	2	3
*	*	4	5	6	3	3	3	3	3
*	*	4	*	7	4	7	3	3	*
*	*	5	*	*	4	*	3	3	†
*	*	*	*	*	3	†	4	3	†
*	*	*		*	*	†	*	*	†
††	*	*	†	*	*	†	††	*	†
CF1 mice									
*	1	1	1	1	1	1	1	1	1
*	1	1	1	1	1	1	1	1	1
*	1	3	1	1	1	1	1	1	1
*	1	*	1	1	1	1	2	1	1
*	1	*	1	1	1	1	2	1	1
*	1	*	1	2	1	1	3	2	1
*	2	*	1	*		1	*	2	1
*	2	*	1	*	*	2	*	*	3
*	*	*	*	*	*	*	*	*	*
*	*	†	†	†	†	*	*	*	*

* = Survivor
† = Died in the interval between immunization and challenge.
†† = Intravenous injection of tuberculin unsuccessful.

times are shortest in the *M tuberculosis* group longest in the BCG Prague group, and intermediate in the BCG Copenhagen group

Statistical analysis Using the smallest dose of *M tuberculosis* as basis for comparison, the difference between the groups immunized with 7×10^7 *M tuberculosis* and 1.9×10 BCG Prague is significant (0 1 per cent) The difference between the former group and the group immunized with 3.3×10 BCG Copenhagen is also significant (4.2 per cent) However the differences between the groups immunized with 3.3×10 BCG Copenhagen and 3.8×10^7 BCG Prague, and between the groups immunized with 6.5×10^7 BCG Copenhagen and $7 6 \times 10^7$ BCG Prague, are not significant.

CF1 mice 0.5 mg PPD was a suitable dose for the CF1 mice since there were survivors in all immunized groups, the number of survivors varying from 11 per cent to 67 per cent in the individual groups.

Dose dependence was not seen in any of the groups immunized with each of the three strains. Comparison of the groups receiving the same number of viable units, *i.e.* the groups immunized with 3.8×10^7 BCG Prague, 3.3×10^7 BCG Copenhagen, and 7×10^6 *M tuberculosis* shows that the survival times are not different, but that twice as many animals survived in the BCG Prague group as in the BCG Copenhagen and *M tuberculosis* groups.

Statistical analysis The differences between the groups immunized with 7×10^6

M tuberculosis and 1.9 × 10^7 or 3.8 × 10^7 BCG Prague or with 3.3 × 10 BCG Copenhagen are not significant. Neither are there any significant differences between the groups immunized with 3.3 × 10^7 BCG Copenhagen and 3.8 × 10^6 BCG Prague or between the groups immunized with 6.5 × 10^7 BCG Copenhagen and 7.6 × 10^7 BCG Prague.

DISCUSSION

Tuberculin Shock in the Murids

It has long been generally accepted that tuberculous mice are not sensitive to tuberculin. Not until the early fifties was it reported that injection of a reasonable dose of tuberculin into mice immunized with virulent mycobacteria or BCG could cause death of the animals from shock (*Kirchheimer & Malkiel* 1953)

Whether or not shock can be provoked depends primarily on the strain of mouse, the bacterial strain used for immunization, the immunization dose and route, and the challenge dose and route. The interval between immunization and challenge may also have an influence, particularly when small immunization doses are used.

Strain of mouse It was shown by *Hsu & Weiser* in 1967 that different strains of mice vary in their susceptibility to tuberculin shock. After immunization with 4 mg BCG injected intraperitoneally followed by intraperitoneal challenge with 2 mg PPD C57Bl, NZB and S/W mice developed the greatest sensitivity (100 *per cent mortality*) whereas NZW mice showed almost no response and T6 mice no response at all. Even though the dose of BCG given to the latter animals was increased to 32 mg, only a just demonstrable degree of sensitivity was obtained. The present study provides no indication of where the CF1 mice should be placed in the series of the above-mentioned strains, since these animals were immunized as well as challenged intravenously It can merely be stated that CF1 mice are highly susceptible to tuberculin shock induced by PPD under the present experimental conditions.

Mycobacterial strain While only large doses (several mg) of BCG provoke shock sensitivity in the mouse, *Gray & Jennings* (1955) found that even quite small doses of virulent tubercle bacilli were effective. Doses of from 25 to 450 viable units injected intranasally caused shock in almost all the animals in a group after challenge with 0.2 ml Old Tuberculin. Within the ranges of dosage used in the present study the shock sensitivity of CF1 mice was not greater in the groups immunized with *M tuberculosis* than in those given BCG but a difference would possibly be revealed if the doses were reduced.

Immunization dose A direct correlation between shock sensitivity and dose was found by *Hsu & Weiser* (1967) in experiments where C57Bl mice were immunized with varying doses of viable BCG 4 mg BCG was the smallest dose to cause shock in 100 per cent of the animals. *Dietrich et al* (1962) found that the use of two doses instead of one single dose for immunization provoked a higher degree of shock sensitivity in CF1 mice. One dose of BCG (approximately 2 × 10^6 v.u.) gave no response, but two doses, injected at an interval of 19 days, caused considerable sensitivity Subsequent experiments (*Dietrich et al.* 1974) in which about 600 Swiss albino mice were injected intraperitoneally with 0.1 mg BCG and, 14 days later intravenously with 0.3 mg BCG showed that the average incidence of lethal shock after challenge with PPD 8–9 days after the second injection of BCG was 83 ± 16 per cent. *In the present study the shock sensitivity* was not correlated with the dose, either after immunization with BCG or with *M tuberculosis* even though the dose of the latter strain varied from 7 × 10^4 to 7 × 10^7

It was shown, however that a single dose of BCG or *M tuberculosis* administered intravenously was sufficient to provoke a high shock sensitivity This is not in contrast to the findings of *Dietrich et al.* (1962) in which the tuberculin was injected intraperitoneally

Route of immunization BCG or virulent mycobacteria are the most effective when

injected intraperitoneally or intravenously if given subcutaneously they have only poor effect (*Dietrich et al* 1962 *Han & Weiser* 1967)

Dose of tuberculin and route of injection The release of shock is conditioned by the size of the tuberculin dose and the route of injection. *Han & Weiser* (1967) who injected varying doses of PPD intraperitoneally into groups of mice immunized with BCG found that the number of deaths from shock increased the higher the dose and that the lowest lethal dose was 1 mg It applies also to tuberculin that the effect is greater after intravenous injection than after intraperitoneal or subcutaneous injection. *Dietrich et al.* (1962) could not provoke shock in CF1 mice immunized with a large dose of BCG and challenged intraperitoneally with 1 mg PPD (RT 23) It was only in experiments where immunization was carried out with two doses of BCG or a strain of *M bovis* that the lethal effect exceeded 50 per cent. In the present study 2 mg PPD injected intravenously killed almost 100 per cent of the CF1 mice immunized with a single dose of BCG or *M tuberculosis* while 0.5 mg PPD killed about 75 per cent

Tuberculin Shock in Red Mice

The experimental conditions in the present study were determined on the basis of the results obtained in mice of the murid family In order to obtain the greatest possible shock sensitivity the animals were immunized intravenously with doses of about 1 mg BCG and with doses of *M tuberculosis* the largest of which was just below the minimum lethal dose. Challenge was performed in half of the animals 3 weeks after immunization with 2 mg PPD injected intravenously Since this proved to be too strong a dose, at any rate for the CF1 mice, the rest of the mice were challenged 1 week later with 0.5 mg PPD also injected intravenously This dose of tuberculin, combined with the doses chosen for immunization, was suitable for the demonstration of a difference, or a lack of difference, between the ability of the individual strains to provoke shock sensitivity both in red mice and CF1 mice.

None of the non immunized red mice or CF1 mice died after challenge with the doses of tuberculin used. On the other hand, the immunized red mice were strongly sensitive to tuberculin though not as sensitive as the CF1 mice, nearly all of which died within the first 24 hours after challenge with 2 mg PPD However the difference was not particularly marked, as can be seen from Expt. 4, where 27 out of 81 red mice and 25 out of 86 CF1 mice died after challenge with 0.5 mg PPD

The strain of *M tuberculosis* provoked greater shock sensitivity in the red mice than the BCG strains and, of the latter BCG Copenhagen gave a slightly stronger sensitivity than BCG Prague. The difference between the effect of *M tuberculosis* and the BCG strains was significant, whereas the mutual difference between the BCG strains was not significant. In contrast, in the CF1 mice no difference in ability of the strains to provoke shock sensitivity could be demonstrated, not even a difference between the weakest BCG strain and *M tuberculosis*.

It can be concluded from the experiments that red mice are more suitable than CF1 mice for demonstrating degrees of difference in the systemic, delayed hypersensitivity provoked by mycobacterial strains of varying virulence.

The writer is grateful t *Michael Weis Bentzon* Chief of the Biostatistical Department, Statens Seruminstitut, Copenhagen, who has performed and described the statistical analysis.

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THE EFFECT OF ASCORBIC ACID ON PRODUCTION OF HUMAN INTERFERON AND THE ANTIVIRAL ACTIVITY *IN VITRO*

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Dahl, H. & Degré, M. The effect of ascorbic acid on production of human interferon and the antiviral activity *in vitro* Acta path microbiol. scand. Sect. B, 84 280–284 1976.

The effects of ascorbic acid on interferon production and on the antiviral effect of interferon in cultures of human cells were investigated. Ascorbic acid enhanced the interferon levels produced by human embryo skin and human embryo lung fibroblasts, induced by Newcastle disease virus and by polyinosinic-polycytidylic acid. The same concentrations of ascorbic acid had no effect on interferon production in two lymphoblastoid cell lines induced by Sendai virus. Leucocyte interferon assayed in lung fibroblasts titrated 0.2–0.3 $\log_{e}$ units higher in the presence of 5 μg ascorbic acid than in the absence of the latter

Key words Human interferon ascorbic acid.

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One of the most debated medical topics during the last few years is the potential effects of high intake of ascorbic acid. The notion, strongly and consistently advocated by *Pauling* (11 12) is that intake of ascorbic acid in large doses, that is doses ranging from 1 to 5 gram per day has a favourable influence on the incidence and development of common cold, the most common of all human infectious diseases, besides its numerous other beneficial effects. The hypothesis is supported by some authors, but strongly challenged by others (9)

Several alternative mechanisms are suggested by which ascorbic acid could act against the common cold, possibly acting in parallel (12) These include direct antiviral effect, stimulation of the phagocytizing leukocytes, immunological enhancement and an action through the interferon system. In the present communication we report our studies of the effect of ascorbic acid on production and antiviral effect of interferon in cultured human cells.

MATERIALS AND METHODS

Cells Fibroblast cells from human embryo lung (HEL) and human embryo skin (HES) were prepared in our laboratory They were grown in Eagle medium and medium 199 50 per cent each with Hanks' salts and with 10 per cent calf serum, and maintained with 5 per cent calf serum. All experiments were performed on cells between 5–15 passages.

P_3HR_1 and EB_4 lymphoblastoid lines were given to us by Dr *E. Tjøtta*, National Institute of Public Health, Oslo. They were grown in suspension cultures in the same medium as described above or

cept that foetal bovine serum was added. None of the cells produced interferon spontaneously

Viruses Sendai strain of para-influenza 1 virus and Newcastle disease virus (NDV) were grown in the allantoic cavity of 9 days old embryonated hens eggs. Allantoic fluids containing 512 or more haemagglutinating units, tested with guinea pig erythrocytes, were pooled and kept at −20° C.

Vesicular stomatitis virus (VSV) Indian strain, was grown in L-F mouse fibroblast cells. Two days after inoculation, when the cytopathogenic effect was practically complete the cultures were frozen and thawed, and the supernatant filtered through Millipore filter 0 45 μ pore diameter The samples were kept at −20 C. They were titrated on $L\text{-}F_1$ cells by the infectivity end point micromethod.

Chemicals Sodium salt of l-ascorbic acid was obtained from Sigma Chemicals Co. Standard stock solution in distilled water containing 10 mg per ml, was kept at −20 C and fresh dilution was made for each experiment. Polyinosinic acid and polycytidylic acid were bought from Miles Laboratories Inc. as separate homopolymers. Equimolar solution was prepared in sodium phosphate buffer (pH 7 4) as indicated by *Field et al.* (6) Hypochromic effect (30 per cent) at 244 nm indicated formation of the complex poly I:poly C, which was kept in stock solution at −20° C, and thawed immediately before use. Cycloheximide and Actinomycin D were purchased from Sigma Chemicals Co.

Induction of interferon (IF) production. Suspension cultures were induced with Sendai virus similarly to the well-established method of human leukocyte induction. On the other hand, fibroblast cultures are known to be good interferon producers when stimulated with NDV

P HR$_1$ and EB cells, ca 5 × 10^7 cells in 1 ml medium were added 0 5 ml Sendai virus containing 1024 haemagglutinating units per ml. Ascorbic acid was added simultaneously in concentrations indicated under Results. The tubes were incubated in a roller for 20 hours, then sedimented by centrifugation at 3500 rev/min for 10 minutes. The supernatant was adjusted to pH 2 by HCl in order to destroy the inducing virus. After 2 days, the samples were neutralized by NaOH.

HEL and HES cells were induced with poly I poly C according to the procedure described by *Billiau et al.* (1) Briefly confluent monolayers were "primed" with 100 units of IF overnight. Next day they were induced with poly I·poly C, 50 μg/ml medium for 1 hour at 37 C. After removal of the inducer the cells were treated with Cycloheximide 10 μg/ml for 4 hours and then with Actinomycin D 1 μg/ml for 2 hours. The supernatants after 24 hours incubation were clarified by centrifugation and tested for interferon activity Ascorbic acid was added both during the priming period and during the induction period.

HEL and HES cell monolayers were induced with NDV 1024 HA units per ml. After 90 minutes adsorption, the cells were washed and fresh medium was added. Ascorbic acid was added to the medium during both phases of induction. The supernatant was separated after incubation over night and it was pH 2 treated as described above.

Assay of interferon activity Interferon activity was tested by the infectivity inhibition microtest (5) Briefly twofold dilutions of the samples were added to freshly seeded HEL cells. After incubation overnight, the test cups and the control cups were challenged with approximately 10 50 per cent tissue culture infectious doses ($TCID_{50}$) of VSV A back titration of the challenge virus was run in parallel in each titration. Microscopical reading was done after 3 days incubation at 37 C when the end point titration of VSV was complete. IF titre was estimated as the highest dilution which inhibited viral cytopathogenic effect in 50 per cent of the cups, calculated by the method of Reed and Muench. The titres were calibrated to 10 $TCID_{50}$ of challenge virus by means of the standard slope of regression line for human IF tested in the HEL/VSV system, as described in detail elsewhere (4) One unit of the standard preparation 69/19 is equivalent to one unit in our system. To assay the effect of ascorbic acid on the antiviral activity of IF ascorbic acid was added to the test cups simultaneously with IF in concentrations indicated under Results.

RESULTS

Toxicity of Ascorbic Acid on Human Fibroblast Cells

HEL and HES cells were grown in the presence of various concentrations of ascorbic acid. The cultures were examined micro-

TABLE 1 *Observed Toxic Effect on Human Embryo Lung Cells Grown in Constant Presence of Ascorbic Acid*

Concentration of ascorbic acid	Toxic effect after incubation for	
	2 days	4 days
0 μg/ml	—	—
10	—	—
25	—	+
50	—	+
100	—	+ +
200	+	+ +

— normal cell monolayer + some degenerated cells + + most or all cells degenerated.

TABLE 2. *Interferon Levels Produced by NDV Virus in HEL and HES Cells in the Presence of Various Concentrations of Ascorbic Acid*

Concentration of ascorbic acid	Interferon titre ($\log_{10}$)	
	HEL cells	HES cells
0 µg/ml	2.25	1.90
10	2.65	2.30
20	2.65	2.30
30	ND	2.30
40 "	ND	2.20
50	2.45	2.10
100 "	2.55	ND

scopically on days 2 and 4 for signs of toxic effect. Table 1 shows that no toxicity was observed if 10 µg per ml or less ascorbic acid were used a slight or moderate toxic effect (the majority of cells were normal) would be obtained with 25 and 50 µg per ml, and a marked toxic effect (majority of the cells degenerated or dead after 4 days) in the presence of 100 µg ascorbic acid or more per ml. Addition of ascorbic acid up to 50 µg per ml, did not influence the production of infectious VSV

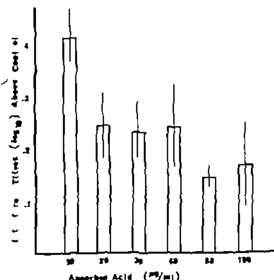


Fig 1 Interferon production in human embryo lung fibroblasts induced by Newcastle disease virus in the presence of various concentrations of ascorbic acid. Mean of 7 experiments. Vertical bars indicate 1 S.D

Effect of Ascorbic Acid on Interferon Production

Induction of HEL and HES cells with NDV resulted in production of comparable levels of interferon. Ascorbic acid in various concentrations was added to the system. A typical experiment with both cell types is shown on Table 2. The addition of ascorbic acid to the inducing system resulted in a slight elevation of the interferon titres produced by both cells. This trend was highly reproducible Fig 1 summarizes the results of 7 induction experiments. All concentrations, including the toxic (100 µg per ml) and subtoxic (20 and 50 µg per ml) concentrations of ascorbic acid, enhanced interferon production. Maximal effect was observed in the presence of the lowest dose tested (10 µg per ml) As the differences exceed three standard deviations they are considered significant.

IF induction in HEL and HES cells with poly I·poly C also gave comparable results. Addition of ascorbic acid generally enhanced the IF levels similarly to the enhancement observed after NDV stimulation. However the results of repeated experiments varied considerably and thus, any clear-cut dose response relation could not be exposed in 10 repeated experiments.

If stimulated with Sendai virus, P HR$_1$ cells are fairly good IF producers while EB cells are rather poor The IF levels produced by these cells were not altered by addition of ascorbic acid in concentrations up to 100 µg per ml. Ascorbic acid alone did not stimulate interferon production or release in any of the cells.

Effect of Asborbic Acid on the Antiviral Activity of Interferon

A standard leukocyte interferon preparation, given to us by Dr *Kari Cantell*, Helsinki, was repeatedly titrated for antiviral activity levels in the presence of various concentrations of ascorbic acid in order to study whether ascorbic acid influences the effector side of the interferon system. Results of 5 ex

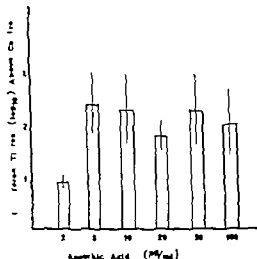


Fig. 2 Antiviral activity of human leukocyte interferon against vesicular stomatitis virus in human embryo lung fibroblasts in the presence of various concentrations of ascorbic acid. Mean of 5 experiments. Vertical bars indicate 1 S.D

periments are summarized in Fig. 2 and a typical experiment is shown in Table 3. By addition of 5 µg per ml of ascorbic acid to the culture medium, the IF preparation titrated 0.2–0.4 $\log_{10}$ units higher than without ascorbic acid. Further increase of the concentration did not further enhance the titre. Extremely small concentrations of ascorbic acid, those carried in the samples induced in the presence of the drug, did not influence the antiviral titre.

DISCUSSION

The results of these experiments suggest that ascorbic acid may enhance the interferon production in human cells, comparable with the reported enhancement in murine cells, primary mouse embryo fibroblast and mouse L cell cultures (13, 14). This effect is apparently not dependent on the nature of inducer. Both virus-induced interferon and synthetic nucleic acid-induced interferon were enhanced by the addition of ascorbic acid to fibroblast cultures. On the other hand, the same treatment of lymphoblastoid cells did not influence the interferon production by

TABLE 3 *Antiviral Titres of Interferon Assayed in HEL Cells in the Presence of Various Concentrations of Ascorbic Acid*

Concentration of ascorbic acid	Interferon titre ($\log_{10}$)
0 µg/ml	2.25
5	2.65
10	2.63
25	2.65
50	2.43
100	2.35

Sendai virus. It may be of significance in this connection to point to the recent findings of at least 2 distinctly different interferon species produced by human fibroblast cells and leukocytes (8, 15). The interferon production of lymphoblastoid cells is considered to be of the same pattern as that of leukocytes. The different effect of ascorbic acid on fibroblast and lymphoblastoid cells may be a function of this heterogenity and reflect a difference in the production pattern or release mechanisms in various cell types.

Ascorbic acid may act at several sites. It may influence the interaction of the inducer with the cell surface or within the cell (2). It may influence the production or the stability of interferon messenger RNA (16). Theoretically ascorbic acid should increase protein biosynthesis and biosynthesis of cyclic AMP (10). Cyclic AMP is reported to stimulate the antiviral activity of interferon (7, 17). At the present time we can only guess which one of these or other possible mechanisms are involved.

Whether the enhanced interferon production is of any significance at the level of the macroorganism cannot be stated for certain on the basis of our knowledge. In murine experiments, ascorbic acid enhanced interferon response both in cell cultures and in the mouse (13, 14). To our knowledge there are no convincing experimental studies showing an increased resistance of macroorganisms against viral infections. Quite naturally, the human organism has received much attention in this respect. Numerous reports on the clin-

ical efficacy of ascorbic acid on the common cold have appeared (11 3 9) Several authors found that the incidence and duration of illness would be reduced, but some of these studies were poorly controlled and not scientifically evaluated. Others found no differences between groups of patients treated with ascorbic acid and placebo, respectively In a review of 14 clinical trials (3) it is concluded that the minor benefits of questionable validity are not worth the potential risk, however small that might be.

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HUMAN INTERFERON AND CELL GROWTH INHIBITION

I Inhibitory Effect of Human Interferon on the Growth Rate of Cultured Human Cells

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Dahl, H. & Degré M. Human interferon and cell growth inhibition. I. Inhibitory effect of human interferon on the growth rate of cultured human cells. Acta path. microbiol. scand. Sect. B 84 285–292, 1976.

Human interferon inhibited the growth rate of cultured human cells. These included diploid fibroblast cells from embryonal lung and skin, established lines of HeLa and U-amnion cells, all grown in monolayer; and the established lymphoblastoid line P3HR1 grown in suspension. Cells growing fast were inhibited to a higher degree than those growing slowly The inhibitory effect was dose dependent but the dose-dependency was different in various cell types. The inhibitory effect of leukocyte interferon and fibroblast interferon was quantitatively comparable.

Key words Human interferon cell growth inhibition.

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In addition to their well known antiviral activity interferon (IF) preparations exhibit a variety of biological effects. These effects include inhibition of the growth rate of homologous, malignant and normal cells (8, 12, 20) enhancement of the cytotoxic effect of double stranded RNA (23 24) and of sensitized lymphocytes (19) enhancement of phagocytosis by macrophages (15 16) enhancement of interferon production by "priming" (21 22) a complex effect both on primary and secondary antibody response (5 10) etc.

The majority of these investigations have been concerned with the murine system. Much less is known about the non-antiviral effects of human IF It is reasonable to assume that, in spite of some differences between the murine and the human IF systems, mainly in their physico-chemical characteristics (25) their biological effect and mode of action are identical in principle. However as human IF currently is used in clinical trials, it is of increasing interest to investigate more thoroughly the nature and significance of the various non-antiviral activities in the human system to confirm the general validity of these biological principles. Some data (2 4 9 14 18) mostly data on transformed human cells, indicate a close similarity to the murine system. The published results, however are by far too scanty to allow a generalization applying also to the human system.

One of the non-antiviral effects most thoroughly investigated and probably the most important effect, is the effect of IF on cell growth, since it may have a direct significance for the antitumour effect of IF We decided to study more closely the growth inhibitory effect of human IF In the present paper the effect of different human IF preparations on the growth of various human cell types is reported.

MATERIALS AND METHODS

Cells Lung cells (HEL) and skin cells (HES) from human embryo were prepared from a 16 weeks old embryo. In all experiments reported in this study HEL and HES cells were used in passages 4–12. HeLa cells were received from Dr *I Orstavik Ullevål Hospital, Oslo. The* U-line of human amnion cells (U-cells) was kindly given to us by Dr *K Cantell*, State Serum Institute, Helsinki, Finland, and the lymphoblastoid cell line producing EB [illegible] P3HR1 was provided by Dr *E Tjøtta* N tional Institute for Public Health, Oslo. The continuous line of mouse fibroblasts, $L\text{-}F_1$ was given to us by Dr *S Haahr* Aarhus University Denmark.

All cells were seeded in a mixture (50:50) of Eagle s minimum essential medium with Hank's salts (EH, Grand Island Biological Company New York (GIBCO)) and Medium 199 (GIBCO) it was supplemented with 10 per cent inactivated calf serum, 0 044 per cent NaHCO and tetracyclin, and maintained in the same medium with per cent calf serum and 0 132 per cent $NaHCO_3$. not otherwise stated in the text, all tests for ntiviral and cell growth inhibitory activities were un in the same med um with 2 per cent calf erum and 0 132 per cent $NaHCO_3$.

Viruses Sendai virus was grown in the allantoic avity of embryonated hens eggs. Allantoic fluids with haemagglutination (HA) titres ≥ 1024 were ooled and stored in small volumes at —20° C. esicular stomatitis irus (VSV) was grown in !-day-old cultures of L F cells. When the cytoathogenic effect was complete, the supernatant fter freezing and thawing of the cultures was ollected. Virus was stored in 0.5 ml ampoules t —70 C a fresh ampoule was used in each experiment. Samples of VSV were assayed by the inectivity end point titration method performed in nicrotrays with the various cells.

Chemicals Polyinosinic and polycytidylic acids n the form of monopolymers were obtained from Miles Laboratories, Inc. they were dissolved in).06 M phosphate buffered salt sol tion (PBS) to a concentration of 1 mM P per ml, sterilized by filtration and mixed for 1 hour at room temperature. Formation of the double strand (Poly I·C) was proved by an about 30 per cent hypochromic effect, measured at 233 nm in a Hitachi spectrophotometer Before use, Poly I·C was heated to 90 C and allowed to cool slowly to ensure maximal formation of double strands. Cycloheximid obtained from SIGMA Chemical Company was dissolved in PBS to a final concentration of 1 mg per ml and stored at 4 C. Actinomycin D (SIGMA) was stored at —20 C as a stock solution containing 1 mg per ml in 96 per cent alcohol. Before use it was further diluted in PBS to contain 100 μg per ml.

Production of human leukocyte interferon Human leukocyte IF was prepared essentially as described by *Cantell et al.* (5) Heparinized whole blood from healthy donors was mixed with 1/5 volume of a 6 per cent Dextran 250 solution and the red cells were allowed to sediment for 1 hour at 37 C. The leukocyte-rich plasma was then pipetted off and EDTA was added to a final concentration of 0 05 per cent. The pH was adjusted to 7 6 and the leukocyte suspension was placed at 2–4 C overnight. On the following day the plasma was decanted and the sedimented leukocytes were suspended in 9 volumes of 0 83 per cent ice cold NH Cl to lyse residual red cells. After 10 minutes at 0° C, the leukocytes were centrifuged at 500 × g for 10 minutes they were resuspended in medium (199 containing 4 per cent $(NH_4)_2SO$ -precipitated human serum (11) and 3 mg of TRICINE (SIGMA) per ml) The cells were counted and adjusted to 10^7 cells per ml before "priming" with 100 units of IF per ml for 1 hour and subsequently induced with 500 HA units of Sendai virus per ml After incubation at 37 C for 22 hours, the IF containing medium was harvested by centrifugation at 2000 × g for 30 minutes. The virus was inactivated by treatment of the crude IF t pH 2 for 2 days t 4 C before storage at —20 C.

Production of human fibroblast interferon Human fibroblast IF was prepared by the super induction method described by *Billiau t al.* (1) HES cells were grown in roller bottles for at least 1 week after confluency was obtained. Medium containing 50 μg of Poly I·C per ml was added to the cultures. After 1 hour the cultures were washed 3 times and supplied with medium containing 10 μg of cycloheximid per ml. After further incubation for 1 hour 1 μg of actinomycin D was added per ml and the cultures were again incubated for 3½ hours. The medium containing the metabolic inhibitors was removed the cultures were washed 3 times and incubated overnight in fresh medium. The next day the IF containing supernatant was harvested, treated at pH for 2 days and stored at —.0 C.

Test for antiviral activity Antiviral activity was measured by the infectivity inhibition microtest

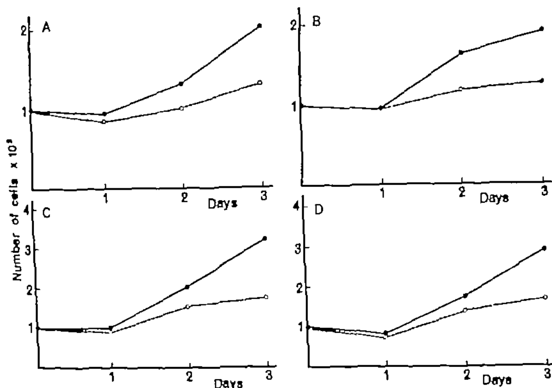


Fig 1 Growth curves for the different human cell types cultiv ted in the presence of interferon.
●—● control without IF
○—○ cells seeded with 100 units of IF per ml.
A HEL cells with leukocyte IF
B: HEL cells with HES-IF
C: HeLa-cells with leukocyte IF
D: U-cells with leukocyte IF
Each point represents the average value from at least 5 parallel cultures.

(6) In short serial dilutions of IF were prepared in microtrays (Linbro IS-FB-96, Linbro Chemical Co., Inc.) in 0 1 ml of medium. To each cup, 20,000 HEL cells were added in 0.1 ml of medium Control cups were seeded with 20,000 HEL cells in 0.2 ml of medium. The trays were incubated overnight at 37 C in 5 per cent CO -atmosphere before the addition of 10 $TCID_{50}$ of VSV in 0 025 ml of medium. A back titration of the challenge virus was run simultaneously After further incubation at 37 C in 5 per cent CO_2 atmosphere for 3 days, the trays were read microscopically for inhibition of development of CPE The titre was determined as the dilution which inhibited the CPE in 50 per cent of the cups, calculated by the method of *R d & Muench* A laboratory standard of human leukocyte IF kindly supplied by Dr A *Cantell* was tested in parallel all titres stated were adjusted to the international standard preparation 69/19 (National Institute for Medical Research, Mill Hill, London)

Test for cell growth inhibition. Culture tubes were seeded with 10^5 cells in 1 ml of medium containing the indicated amount of IF Control tubes were seeded in medium without IF The cultures were incubated at 37 C in 5 per cent CO atmosphere. At the indicated time, the monolayers were treated with trypsin-versene and the cells were counted in a haemocytometer Each po t in the curves represents the mean alue of t least 5 parallel cultures.

RESULTS

Preliminary experiments confirmed our assumption that human IF preparations reduce the growth rate of several types of homolo-

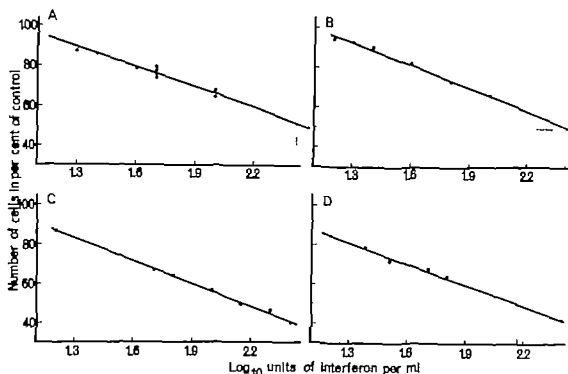


Fig. 2. Dose-response curves for the effect of interferon on the growth of different human cells.
A: HEL cells with leukocyte IF
B: HEL cells with HES-IF
C: HeLa cells with leukocyte IF
D: U-cells with leukocyte IF
Each point represents the average value from at least 5 parallel cultures.
The dotted lines indicate the amount of IF necessary for a 50 per cent reduction in cell count.

, ıa cells. As regards the further experiments, he growth conditions had to be strictly tandardized. This was the reason why one ind the same type of medium was used for he different cells. It is well-known that the erum concentration in the medium will in luence the growth rate. Accordingly we in restigated whether various serum concentra- ions might have some influence on the IF effect on the growth rate. HEL cells were grown in medium containing 2, 4, 6, 8 and 10 per cent calf serum. To half of the tubes were added 100 units of IF. After 3 days, the cultures were treated with trypsin-versene and the cells were counted. The number of cells in all cultures containing IF were reduced to the same extent, by approximately 30 per cent as compared with the controls. The reduction was not dependent on the serum concentration. As the cultivation of fast growing HeLa and U-cells required a change of medium when higher concentrations of serum were used, 2 per cent was chosen for all further experiments. Since further cultivation of the same cells after 3 days also required a change of medium, the subsequent grov experiments were terminated on the th day.

Effect of Different Human Interferon Preparations on the Growth of Human in Monolayer Cultures

HEL, HeLa and U-cells were seeded presence of either leukocyte IF or fi- IF, 100 units per ml, as described

TABLE 1 *The Susceptibility of the Different Types of Human Cells to the Inhibitory Effect on Cell Growth and th Antiviral Activity of Human Interferon*

Cells	Cell count* with serum per cent		Susceptibility to	
	2	10	Antiviral effect‖	Growth inhibitory effect†
HEL	165	194	1	260
HeLa	323	n.d.§	17	145
U-cells	290	n.d.	2	145
P3HR1	n.d.	157	n.d.	600

* *Number of cells* × 10^3 *after 5 days of cultivation in the presence of the indicated concentration of serum.*
‖ Number of interferon units required to cause a 50 per cent protection against viral attack.
† Number of interferon units required to reduce the cell count by 50 per cent.
§ *Not done.*

Materials and Methods. *Each day 5 IF* treated tubes and 5 control tubes were treated with trypsin-versene and the cells were counted. The medium was not changed during the incubation period. Representative growth curves are shown in Fig 1. In each experiment, the growth rate of the cells was reduced in the presence of IF. The two different IF preparations, made in leukocytes or fibroblast cells, gave comparable reduction of HEL cells.

The degree of growth reduction by 100 units of IF differed to a certain degree in the three cell types. After three days of incubation, the number of IF treated HEL cells was reduced by 35 per cent, U-cells by 39 per cent and HeLa cells by 41 per cent, as compared with the controls. This tendency was consistent.

To further investigate this phenomenon, the influence of the dose of IF on the growth rate was more closely examined.

Dose Dependence of Interferon Effect on Growth of Human Cells in Monolayers

HEL, HeLa and U-cells were seeded in the presence of various concentrations of either leukocyte IF or fibroblast IF. *After 3 days of* incubation, all cultures were treated with trypsin-versene and the number of cells was counted. The results of these experiments are shown in Fig 2. Each point represents the mean of at least 5 cultures.

In all the systems tested, a straight line correlation between the number of cells in per cent of the control and log_{10} units of IF per ml was found. However the susceptibility to the antiviral and cell growth inhibitory activities of IF of the different cells varied considerably (Table 1). If a 50 per cent reduction of HEL cells were to be obtained, 260 units of IF were required necessary as compared with 145 units in the cases of HeLa and U-cells. In contrast, 50 per cent protection against viral infection, as defined by morphological signs of cytopathogenic effect, using 10 $TCID_{50}$ of VSV was obtained by 1 unit of IF in HEL and HES cells, 2 units in U-cells and 17 units in HeLa cells. Thus, if tested in different cell systems, the two activities seem not to be correlated to each other

Effect of Human Leukocyte Interferon on the Growth Rate of the Lymphoblastoid Cell Line P3HR1 in Suspension Cultures

The effect of human leukocyte IF on the growth rate of P3HR1 cells was tested in suspension cultures seeded with 10^5 cells per ml of medium containing 10 per cent inactivated calf serum and the indicated concentrations of IF (Fig. 3). The cultures were incubated at 37 °C in 5 per cent CO_2-atmosphere and counted every day. Each point in the figure represents the mean value of at least 4 separate counts.

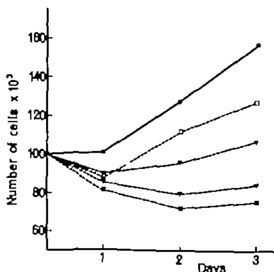


Fig 3 The effect of human leukocyte interferon on the growth of P3HR1 cells.
The cells were cultivated in medium containing 2.1 □—□, 2.4 ▼—▼ 2.7 ▽—▽ or 3.0 ■—■ $\log_{10}$ units of IF per ml.
●—● control without IF
Each point represents the average val from at least 4 independent countings of the same suspension.

As shown in the figure the presence of human leukocyte IF in the medium resulted in a dose- and time-dependent inhibitory effect on growth, comparable with that found in monolayer cultures. If however a 50 per cent reduction in cell count had to be obtained after 3 days of incubation, 600 units of IF had to be added per ml of medium (Table 1)

DISCUSSION

The results presented in the present paper confirm earlier observations (2, 4 9 18 19) according to which IF preparations actually inhibit the growth of homologous cells in culture. The origin and nature of these cells are highly different. HEL and HES cells are slowly growing, presumably normal diploid fibroblasts growing in monolayers. HeLa and U-cells are well-established lines as HeLa cells are derived from cervix cancer and U-cells derved from amnion obtained at a normal delivery both are of epitheloid origin and growth in monolayers is relatively rapid. P3HR1 cells are derived from an established lymphoblastoid cell line growing slowly in suspension cultures. The fact that such varied collection of cells is so similarly affected suggests that the inhibitory effect of IF on homologous cells is a rather general phenomenon. However negative results have also been reported (9 *Strander* personal communication) Differences in experimental conditions may to a certain degree explain this discrepancy Further results are needed before a general conclusion concerning the effect of human IF on cell growth can be drawn.

It applies to all cells tested that correlation between per cent reduction in cell counts and $\log_{10}$ units of IF used per ml followed a straight line. However the extent to which growth of the various cells was reduced differed. Such reduction seems to be more marked in the case of rapidly growing cells such as HeLa and U cells, than in the more slowly growing cells as HEL and HES cells. This observation is in agreement with the earlier report by *Lee et al.* (18) It goes without saying that other factors which may have influence on the susceptibility of cells to the growth depressing effect of IF cannot be excluded for the time being In this connection it may be borne in mind that cell growth enhanced by addition of higher serum concentrations failed to alter the extent to which growth was reduced by IF

Several recent reports (13 17) indicate that human IF preparations produced in leukocytes and in fibroblasts differ in several respects. Therefore it is interesting to note that the reduction in growth was dose-dependent to the same extent whether leukocyte IF or fibroblast IF were used. This indicates that the antiviral activity and the growth depressing activity of both are of approximately the same mutual proportions. This also argues against the notion that growth depression might be a result of toxic impurities, bearing in mind that the two preparations were produced by so different techniques that it is improbable that they should contain the same impurities.

The results indicate that the sensitivity to the antiviral effect and to the growth inhibitory effect varies independently in different cell types treated with the same IF preparations. The reason for this lack of correlation is difficult to explain, but it may be due to cellular differences. The regulating systems which control the growth rate and the induction of the antiviral state in the different cells may vary in sensitivity to the effects of IF or the receptor sites or transport systems of cell membranes may differ. Preliminary results (7) indicate that the two activities can be physically separated. These results allow further investigations on the kinetics and the nature of the two activities.

The growth rate of the lymphoblastoid cell line, P3HR1 was only modestly affected by the leukocyte IF preparation, comparable with findings reported by *Hilfenhaus & Karges* (14). Ten per cent serum was needed for cultivation of these cells and even so, they grow rather slowly. As regards P3HR1 cells, we have no data on their susceptibility to the antiviral activity of IF; still, they were the least sensitive to the growth reducing activity which is in agreement with our suggestion that this effect somehow is correlated with the growth rate.

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Acta path. microbiol. scand. Sect. B 84 293–299 1976

IDENTIFICATION OF ANTIGENS WITHIN THE SO CALLED VACCINIA L-S COMPLEX BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORESIS

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Prante, P. H., Haukenes, G. & Gurvin, L. Identification of antigens within the so-called vaccinia L-S complex by means of quantitative immunoelectrophoresis. Acta path. microbiol. scand. Sect. B, 84: 293–299 1976.

The so-called L-S antigen complex of vaccinia virus antigens was studied using gel diffusion and various immunoelectrophoresis methods. The low resolution power of the gel diffusion method and the inconsistency of the results obtained rendered this method unsuitable for identification of individual antigens and for comparative studies. The sensitivity and simplicity of counter current electrophoresis showed the superiority of this method for rapid diagnosis of pox-virus infections. Simple rocket or line electrophoresis could not be used to distinguish the antigens. The height of the individual rockets and the position of the lines were greatly influenced by the strain of virus and the antibody composition of the antiserum. Crossed and crossed-line electrophoresis gave distinct and reproducible precipitation patterns which may provide a basis for further studies of vaccinia precipitinogens. The presumed L-S antigen complex was composed of two distinct antigens. The L antigen was more negatively charged and showed an approximate S value of 10 as compared to 7S for the S antigen.

Key words: Vaccinia antigens quantitative immunoelectrophoresis.

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By the use of double diffusion in agar up to 20 precipitation lines have been demonstrated with extracts of vaccinia virus-infected cells and anti-vaccinia hyperimmune sera (*Appleyard & Westwood 1964*). Among these lines the so-called L-S antigen complex (*Craigie & Wishart 1936, 1938*) is usually discernible as two major bands about midway between the antigen and antibody basins. They are characterized by their sensitivity to heat, labile (L) and stable (S). In some studies a third line has been distinguished within the L-S complex (*Marquardt et al. 1969*). The other precipitation lines are less well defined, their presence and location being greatly influenced by the host cell used for propagation of the virus and by the antibody composition of the antiserum. *Westwood et al.* (1965) claims that even the L-S antigens are difficult to define and cannot be distinguished from all the other precipitation lines.

Using the standard immunoelectrophoresis technique, *Cohen & Wilcox* (1966) defined several lines by their electrophoretic mobility and their position after the precipitation step.

In the present work we have studied the two dominant precipitinogens in extracts of vaccinia virus-infected HeLa cells. They are most probably the same as those defined by *Cohen & Willcox* (1966) as the L and S antigens. We have used the various quantitative immunoelectrophoresis techniques (*Laurell* 1965, *Axelsen et al.* 1973) in combination with other separation methods.

MATERIALS AND METHODS

Cell Culture

HeLa cells (Bristol strain, calf serum adapted, obtained from Flow Laboratories, Irvine, Scotland) were grown in monolayers, using Eagle's Minimal Essential Medium (MEM) supplemented with 10 per cent calf serum (Bio Cult., Glasgow Scotland) and antibiotics. After infection the serum supplement was changed to 2 per cent.

Monolayers of lung fibroblast were made from homogenised and trypsinised lung tissue from 5-day-old rabbits. Eagle's MEM was supplemented with 20 per cent rabbit serum (Bio-Cult.) Before infection, one passage was made with medium containing 10 per cent serum obtained from the rabbit to be used for production of antiserum.

Vaccinia Virus Strains

The *Copenhagen strain was the Smallpox vaccine* strain from Statens Seruminstitut, Copenhagen, the IHD-J strain was kindly supplied by Dr *Yasuo Ichihashi*, Kyoto, Japan. This latter strain is a haemagglutination positive and cell *fusion negative vaccinia virus mutant*.

Unless otherwise stated the Copenhagen strain was used.

Propagation of Virus

For the preparation of viral precipitinogens, HeLa cells monolayers were infected at high multiplicity (10 p.f.u./cell) and incubated at 37° C for 24 h. For the production of antisera, virus was propagated in rabbit lung fibroblasts as described above.

Preparation of Vaccinia Virus Antigen (VVA)

Infected HeLa cells were harvested by scraping off the monolayer after incubation for 24 h, *i.e.* before any cytopathogenic effect had developed. The harvested cells were frozen and thawed four times, centrifuged at 1,500 × g for 10 min to remove whole cells and large cell debris, and thereafter at 27,000 × g for 1 h to remove virus and particulate substances which do not migrate in agar. The resultant supernatant had a protein content of 1–2 mg/ml.

Antisera

Rabbits were immunized with virus produced in rabbit lung fibroblasts. Infected cells were harvested after incubation for 2 days, and the virus was released by freezing and thawing three times.

One group of rabbits was immunized with infectious virus. An initial dose of 3 × 10^{6} p.f.u. of virus was injected intravenously. Four and 5 weeks later 2.4 × 10 p.f.u. of virus in Freund's incomplete adjuvant were given intramuscularly each time. The antiserum used had vaccinia virus haemagglutination inhibition (cf. below) antibody titre of 256 (serum III).

Antisera against the presumed L and S antigens were raised in rabbits immunized with the material of the 2 precipitation lines obtained by line immunoelectrophoresis. After immunoelectrophoresis the gel was washed with buffer and distilled water before cutting out the 2 lines, about 20 cm long. The obtained gel was frozen and thawed and then homogenized in Freund's complete adjuvant before injection intramuscularly. The rabbits were bled 3–4 weeks after the injection (antisera I and II).

The human antiserum used was a vaccinia immune globulin obtained from Statens Bakteriologiska Laboratorium, Stockholm.

Haemagglutination Inhibition (HAI)

Vaccinia virus HAI antibodies were titrated in WHO perspex trays against 4 haemagglutination units of a purified haemagglutinin preparation.

Double Diffusion in Agar

Basins of 4 mm diameter and 2–3 mm apart were cut in agarose (Indubiose A 37, l'Industrie Biologique Française, S.A., Gennevilliers, France) made up to 1 per cent in saline buffered with phosphate to pH 7.2 (PBS). Precipitation lines were recorded before and after staining (cf. below).

Counter Current Electrophoresis

Holes of 2 mm diameter were cut in a gel containing 1 part of agar and 3 parts of agarose (1 per cent in 0.1 M veronal buffer pH 8.6). The distance between the antigen and antibody basins was 8–10 mm. The electrophoresis was run in a Hepaescreen apparatus (Spectra Biologicals, Oxnard, Calif., USA) at 10 V/cm for 15 to 45 min. The precipitation lines were read without prior staining.

Immunoelectrophoresis

This was run by the standard procedure (*Scheidegger* 1955) at pH 8.2 and 8.6.

Quantitative Immunoelectrophoresis

All experiments were performed with a Shandon Southern U-771 TLE apparatus equipped with tap water cooling. Plates of 10 × 20 cm were covered with agarose. The antiserum was mixed with agarose to a final dilution of 1 in 20. A 1 per cent agarose in 0.05 M veronal buffer pH 8.6, was used throughout.

Crossed Immunoelectrophoresis

Antigen was mixed with an equal volume of 2 per cent melted agarose at 44 C and placed in a 6 mm diameter hole. The electrophoresis was run in the first direction (cf. Fig. 3) for 2 h at 8 V/cm (recorded in the agarose). Thereafter the antiserum-containing agarose was added and the second electrophoresis run into this gel at 2 V/cm for 18 h (second direction).

Line and Crossed Line Immunoelectrophoresis

Ditches 2 mm wide and of varying length were cut in the agarose and filled with antigen in melted agarose as above. The electrophoresis was run as described for the second direction above.

Crossed line immunoelectrophoresis was performed by combining the two procedures above the line serving as a reference.

Staining of Precipitation Lines

The plates were dried by covering the agarose with absorbent paper (Whatman No. 1 covered with soft tissue) under pressure for 15 min. Thereafter the plates were washed twice in PBS, 15 min each time, and placed in distilled water.

The plates were then repeatedly dried by absorbent paper under pressure and finally exposed to heated dry air.

The resulting thin film was stained with 1 per cent Coomassie Brilliant Blue (Serva, Heidelberg, West-Germany) in ethanol-acetic acid-water 45:10:45 by volume. After staining for 15 min the plates were de-stained by the same ethanol-acetic acid mixture.

EXPERIMENTS AND RESULTS

Double Diffusion in Agar

Two major precipitation lines, positioned about midway between the basins, were consistently obtained with different rabbit and human antisera. The lines were however very closely positioned and sometimes almost confluent. Because the relative positions of the lines varied, it was not possible to identify them as L or S lines. Nor did heating at 56 C for 30 min give unequivocal results as both lines could be traced after heating. The presumed L line seemed to be more weakened and diffuse after heating. As a consequence

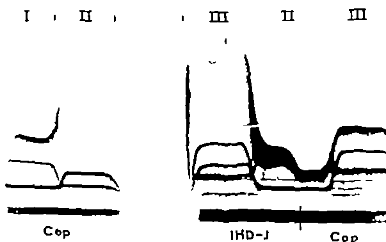


Fig. 1 left) Line immunoelectrophoresis of VVA and sera from 2 rabbits, I and II immunized by the same procedure. Cop: Copenhagen strain. right) Line immunoelectrophoresis of antigens from 2 vaccinia strains (the IHD-J and the Copenhagen strains). I and II Antisera against precipitation lines. III Antiserum against whole lines.

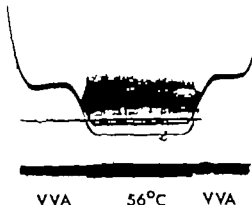


Fig 2 Line immunoelectrophoresis of VVA untreated and treated at 56 C for 30 min.

we had to consider the gel diffusion method as unsuited for further characterization of these precipitinogens.

Counter Current Electrophoresis

As expected, the resolution power of this method was rather poor The sensitivity however was 5 to 10 times that of the double diffusion methods With specimens from vaccinated individuals the precipitation line(s) could be discerned after 15 min. When antigen and antibody were allowed to diffuse for 1 h before electrophoresis it was possible to obtain a reaction of identity with an adjacent reference system

Line Immunoelectrophoresis of Crude VVA

Crude VVA from the two vaccinia strains was examined, and with both of them 2 strong lines were obtained in addition to a number of weaker lines. However the absolute and relative positions of the 2 lines differed greatly As shown in Fig 1 the lines of the 2 strains shifted their mutual position even when examined against the same serum.

The various antisera also differed with regard to absolute and relative amounts of antibodies to the 2 precipitinogens, which led to a shift in position of the lines (Fig 1 a)

Heating of VVA at 56 C for 30 min gave a complex picture (Fig 2) One line became very weak and located nearer to the application line. The other line seemed to be split in two, both lines being nearer to the application line than the lines obtained with untreated VVA. In addition a third line appeared level with the lines in the untreated material. This line was more diffuse and coalescence with the lines of untreated VVA could not be traced.

As a whole, a very good separation of the two major precipitation lines was possible by the use of the line immunoelectrophoresis method. Further identification of the other lines was not feasible.

Crossed and Crossed Line Immunoelectrophoresis

The 2 major precipitinogens showed different electrophoretic mobility in agarose under the conditions employed, the relative migration being 1 4 to 1 (Fig 3)

In order to establish a better reference sys-



Fig 3 Crossed immunoelectrophoresis of VVA.

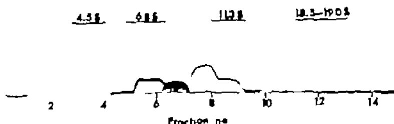


Fig. 4 Line immunoelectrophoresis of fractions from sucrose gradient centrifugation of VVA. The positions of the following proteins with known S values are marked: haemoglobin 4.5S IgG 6.8S, catalase 11.3S and IgM 18.5–19.0S.

tem attempts were made to separate the 2 antigens chemically. This was accomplished by ultracentrifugation of VVA in a 12.5 to 37.5 per cent sucrose gradient together with reference substances of different S values. Line immunoelectrophoresis was performed with each of the 15 fractions obtained. As seen from Fig. 4 2 strong precipitinogens were recovered approximate of 7S and 10S. The 7S and 10S fractions were then used as reference material for the 2 peaks in crossed immunoelectrophoresis, applying the so-called tandem technique (Fig. 5 a and b). The 7S material added a "shoulder" to the first peak whereas the second peak acquired an additional peak from the 10S material.

The 7S and 10S fractions were examined by line immunoelectrophoresis together with untreated VVA and VVA heated at 56° C for 30 min. The 10S antigen was shown to be relatively sensitive to heat (Fig. 6).

The so-called L-S complex accordingly



Fig. 5 Tandem crossed immunoelectrophoresis with VVA and 7S and 10S fractions of VVA from sucrose gradient centrifugation. The 7S and 10S fractions were placed in the respective second holes.



Fig 6 Line immunoelectrophoresis of 7S and 10S fractions from sucrose gradient centrifugation after 30 min incubation in 56 C ([7S] and [10S]) compared with untreated fractions and untreated VVA.

consisted of a 7S and 10S material, the former being more resistant to heat and less negatively charged than the latter

DISCUSSION

Double diffusion in gel has a poor resolution power and the sensitivity is low in regards to the demonstration of precipitinogens and precipitins. Although a great number of lines have been demonstrated by this method with virus antigens and hyperimmune sera, special arrangements of the wells and treatment of the antigens have to be made. The appearance and location of the individual lines are inconsistent, and cannot be used for identification of a particular antigen. As shown here, not even the major L and S antigens are well defined, the lines usually being very closely positioned. Moreover distinction of the two antigens by their different sensitivity to heat was not absolute.

As expected counter electrophoresis showed higher sensitivity for demonstration of antigen, which in addition to the ease and rapidity of performance, makes this method useful in the diagnosis of pox virus infections. Our results are in accordance with those of *Kyriakidou et al* (1973)

Quantitative immunoelectrophoresis methods have been used with success for demonstration of antigenic differences between herpes simplex virus types 1 and 2 (*Jeansson & Vestergaard* 1975) The precipitinogens were well defined according to their electrophoretic migration in the first direction relative to albumin.

In the present study the antigen and antibody concentrations were adjusted so as to be optimal for studying the so-called L-S complex. The 2 major precipitinogens separated well on line and crossed line immunoelectrophoreses.

The L and S antigens are usually referred to as the L-S complex. Our results from ultracentrifugation in a sucrose gradient clearly showed that the 2 antigens represent distinct entities. As both antigens are sensitive to heat to some extent, we prefer at present to designate them according to their approximate S values, 7S and 10S

The 2 major lines were also well separated on crossed immunoelectrophoresis, the 10S material showing the higher electrophoretic mobility

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Acta path. microbiol. scand. Sect. B 84 300-304 1976

IMMUNOCHEMICAL ANALYSIS OF AN UNUSUAL CELL WALL POLYSACCHARIDE FROM ANIMAL COAGULASE-POSITIVE STAPHYLOCOCCI

I Fragments Obtained after Hydrolysis in Hydrofluoric Acid and Alkali

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Endresen, O & Grov A. Immunochemical analysis of an unusual cell wall polysaccharide from animal coagulase positive staphylococci. I Fragments obtained after hydrolysis in hydrofluoric acid and alkali. Acta path. microbiol. scand Sect. B, 84 300-304 1976.

Polysaccharide P (poly P) of canine coagulase-positive staphylococci contains glycerol, glucose, glucosamine, muramic acid, phosphate and the usual peptidoglycan amino acids, but does not cross-react serologically with standard teichoic acids. Products from hydrolyses in hydrofluoric acid and alkali contained phosphates of glycerol and glucose as well as combinations of these, but neither glucosyl-glycerol units nor glucosamine-phosphates were observed. The teichoic acid of poly P is probably a polymer of a repeating unit consisting of alternating glycerol, phosphate and glucose.

Key words: Immunochemistry polysaccharide P animal staphylococci.

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Animal strains of *Staphylococcus aureus* vary with regard to their wall teichoic acids (7 8) In a previous report (2) polysaccharides from pigeon and mink strains (poly H) dog strains (poly P) and swine strains (poly V) were isolated and found to contain glycerol teichoic acids. Whereas poly H was shown to be an ordinary polyglycerolphosphate teichoic acid with N-acetylglucosaminyl substituents, the structures of the two other teichoic acids apparently were somewhat unusual. Neither poly P nor poly V reacted with other sera than homologous ones.

Polysaccharide P been subjected to further immunoch[illegible] ration, and the present report describes the fragments obtained at various hydrolytic conditions.

MATERIALS AND METHODS

Isolation f Polysaccharide

The coagulase-positive *Staphylococcus aureus* dog strain Z14 (2) was selected as the poly P producer The bacteria were grown and extracted (5) and the polysaccharide purified by ion-exchange chromatography and gel filtration as before (2)

A alytical M thods

Acid hydrolysis Samples of polysaccharide (1-2 mg) were hydrolysed with 2 N HCl (0.5 ml) for 3 or 6 h at 100 C in sealed tubes flushed with nitrogen (2) Washed hydrolysates used for quan-

thative analyses by gas-liquid chromatography (GLC) were dissolved in 1 ml of 0.02 M $(NH_4)_2CO_3$, pH 9.5, and 0.2 mg alkaline phosphatase was added (Type II Sigma, USA). After incubation at 37 C for 18 h, the enzyme was removed by heating at 100° C (water-bath) for 15 min and centrifugation. The supernatant was evaporated to dryness at reduced pressure and the carbohydrates prepared for GLC, the whole analytic procedure being that described or referred to in (2). In addition, a column of 3 per cent Poly A 103 on Supelcoport (100–120 mesh, Supelco, USA) (6) was used.

Degradation of polysaccharide by hydrofluoric acid (HF). Poly P (100–300 mg) was incubated with aqueous 60 per cent (w/v) HF at 0° C for 16 h (5) and the mixture then neutralised with LiOH and Li_2CO_3. The LiF formed at 4 C was isolated by centrifugation and washed two times with 2 ml of distilled water. The combined supernatant and washings were concentrated under reduced pressure, one part being tested directly on paper chromatography and the other part applied to a column of DEAE-cellulose (HCO_3^--form). The column was washed with water (200 ml) and then eluted with a linear gradient of $(NH_4)_2CO_3$, pH 8.2 (500 ml of water – 500 ml of 0.4 M $(NH_4)_2CO_3$). One ml from every second fraction (10 ml) was analysed for total phosphorus (13) and the fractions belonging to one peak were pooled and concentrated for further analyses.

Degradation by alkali. Poly P (100–200 mg) was hydrolysed in 1 N NaOH (1 ml) for 3 h at 100° C, applied to a column (5 ml) of Dowex 50 (NH_4^+-form) and eluted with water (100 ml). The eluate was concentrated under reduced pressure and chromatographed on a column of DEAE-cellulose as described above.

Gel filtration of HF- and alkali-degradation products. Samples of hydrolysates were filtered through a column (0.6 × 70 cm) of Bio-Gel P 10 (200–400 mesh, Bio-Rad, USA) in 0.1 M NH_4HCO_3, pH 8.5. The column was calibrated with blue dextrane, raffinose and NaCl. The void volume of the column was 13.5 ml. Fractions (1 ml) were collected and assayed for total phosphorus (13). The low molecular weight fractions were pooled, concentrated and filtered on a column of Bio-Gel P-2 (200–400 mesh).

Analytical paper chromatography was carried out on Whatman No. 1 paper using three solvent systems:

A. Butanol/Pyridine/H_2O (6/4/3 v/v)
B. Ethylacetate/Pyridine/H_2O (40/11/6 v/v)
C. Propanol/NH_3/H_2O (6/3/1 v/v)

The alkaline silver nitrate reagent (12) was used to localize reducing sugars, amino sugars and sugar alcohols. Sugar alcohols were also visualized by sodium metaperiodate-benzidine (11). The periodate-Schiff reagent (9) was applied for α-polyols and the perchloric acid-molybdate-H_2S spray (4) for phosphate esters.

Preparative paper chromatography was performed on Whatman No. 3 MM in solvent C. The sample (*fractions from DEAE cellulose column*) was spotted on a line across the paper and after the run a strip on each side was cut off and stained. Using these markers the areas containing the different substances were cut out and the substances eluted with water.

Enzymatic treatment. Samples of Poly P and subfragments were subjected to α-glucosidase (Sigma) in 0.05 M phosphate buffer pH 6.8 containing 10^{-3} M mercaptoethanol (3), β-glucosidase (Sigma) in 0.05 M acetate buffer pH 5.0 (3) and β-N-acetylglucosaminidase (Sigma) in 0.05 M citrate buffer pH 4.4 (1). The digestions were performed at 37 C for 36 h, a drop of toluene being added beforehand to prevent bacterial growth. The ratio of enzyme to substrate was 1 to 25 in each experiment.

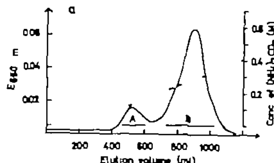
Periodate oxidation was performed in 0.1 M sodium metaperiodate in saline at 20 C in the dark, and *sodium borohydride* reduction as described in (14).

RESULTS

Paper Chromatography of Hydrolysates

Chromatography of the HF hydrolysate in solvent C and spraying with the molybdate spray revealed four spots (Rf 0.42, 0.31–0.25, 0.18, 0.09), the broad spot (Rf 0.31–0.25) being the dominant one. The same four spots were also visible after applying the periodate-benzidine spray, whereas only two purple spots (Rf 0.49 and 0.31–0.25) appeared quickly when the periodate-Schiff reagent was used.

Chromatography of alkali hydrolysate in solvent C also gave four spots with the molybdate and periodate-benzidine reagents (Rf 0.42, 0.33–0.25, 0.16, 0.09). The major broad spot (Rf 0.33–0.25) and the fast moving one (Rf 0.42) were also coloured with the periodate-Schiff reagent. In addition the latter reagent sometimes revealed a weak, pale blue spot with Rf 0.22–0.18. In both hydrolysates, traces of free glycerol were observed, but no free glucose or glucosamine could be demonstrated by the silver nitrate spray. Treatment of the HF hydrolysate with



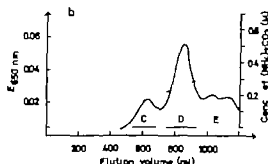


Fig 1 DEAE-cellulose chromatography of hydrofluoric acid (a) and alkali (b) hydrolysates of poly P The curves show the amount of *phosphorus present.*

phosphomonoesterase released some free glu cose, glycerol and about 8 per cent of the phosphate Similar treatment of the alkali hydrolysate released some free glycerol and about 15 per cent of total phosphate.

DEAE-Cellulose Chromatography of Hydrolysates

The elution patterns of HF and alkali-degraded poly P (about 150 mg in each) from the DEAE-cellulose column are illustrated in Fig 1 a and b respectively Trace amounts of free glycerol were observed in the water elutes. The material of the HF hydrolysate was eluted in two peaks (A and B) concentrations of $(NH\ )_2CO$ being 0 11–16 and 0.21–0.33 respectively The alkali degraded material was eluted in three peaks (C, D E) of which only two (C and D) eluted at 0 13–0 18 and 0.22–0.30 M $(NH\ )_2CO_3$, respectively were further ana lysed.

Gel Filtration of Hydrolysates

The gel filtration patterns of HF and alkali-degraded poly P on a column of Bio-Gel P 30 are shown in Fig 2 The elution curves indicate that substances of different size were present. Apparently the HF hydrolysate contained a more uniform low molecu lar weight fraction than the alkali hydrolysate. On the other hand, the HF hydrolysate contained some non-degraded or slightly de graded material which was eluted just after the void volume. Further fractionation on a Bio-Gel P 2 column of the main peaks from the HF hydrolysate (23–28 ml) and the alkali hydrolysate (21–32 ml) showed that most of the material in both instances was eluted just after the void volume A shoulder on the peak from alkali-degraded material indicated differences in size of the organic phosphates. Inorganic phosphate was in both instances eluted in late fractions, the content of this material being highest in the alkali-degraded material.

Chemical Analyses

The organic phosphates separated on DEAE-cellulose (A, B, C, and D) were fur ther analysed by chromatography and frag ments were isolated by preparative paper chromatography Peak A seemed rather

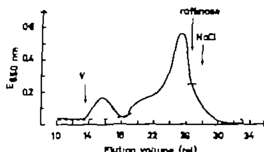


Fig 2 Gel filtration of hydrofluoric acid (——) and alkali (– –) degraded poly P The curves are revealed by determination of phosphorus. V = elution volume of blue dextran.

homogeneous. Chromatography in solvent C revealed one spot with Rf = 0 42, and chemical analysis showed glucose, glycerol and phosphate in the ratio 1 1 1.2 (S_I) No inorganic phosphate was detected after treatment of S_I with phosphomonoesterase. Peak B contained a mixture of polyphosphates, the main spot (S_{II}) with Rf 0.31–0.25 being found to contain glucose, glycerol, glucosamine and phosphate in the ratio 0.9 1 1 0.7 1.8. Some free inorganic phosphate was released by phosphomonoesterase. Free glycerol was also detected, indicating the presence of glycerol monophosphate. S_{II} seemed to be a mixture of two or three different phosphates as judged from the broad spot on chromatography and the unusual ratio of constituents. As with intact poly P all the glucose of both substances (S_I and S_{II}) was destroyed by sodium periodate oxidation. Glycerol was also completely destroyed by periodate in S_I whereas some glycerol was still detectable in S_{II} Reduction by $NaBH_4$ transferred all the glucose of S_I to sorbitol, but only a part of the glucose in S_{II}

Chromatography revealed three and four phosphates in peaks C and D respectively S_{III} (Rf 0.40–42) which was present in both C and D seemed to be similar to S_I containing glucose, glycerol and phosphorus in the ratio 1 1 1.2. Both glucose and glycerol were destroyed by $NaIO_4$. However no glucose was reduced to sorbitol by $NaBH_4$, demonstrating a dissimilarity S_{IV} (Rf = 0.33) also found in both peaks, contained glycerol and phosphate only Phosphomonoesterase released glycerol and inorganic phosphate, showing S_{IV} to be a glycerolmonophosphate S_V (Rf 0.28–0.23) of peak D contained glycerol glucose and phosphate in the ratio 1 1 1 7 The glucose was unaffected by $NaBH_4$, whereas glucose but not glycerol was destroyed by $NaIO_4$. Phosphomonoesterase released about one half of the phosphate as inorganic phosphate. A slow moving substance (Rf = 0.16) which contained glycerol and phosphate in the ratio 1 1.8 was found to be a glyceroldiphosphate. Traces of some other low molecular phosphates present in peaks C and D were not analysed. Glucosamine was not observed in any of the phosphates after alkali-degradation. Digestion of intact poly P or isolated fragments by α-glucosidase, β-glucosidase or β N-acetylglucosaminidase did not result in free sugar Neither could glucosylglycerol be detected by chromatography in solvent A, in which glucosylglycerol is suggested to move just in front of glucose (3)

DISCUSSION

Treatment of polysaccharide P with α and β-glucosidases and β-N acetylglucosaminidase did not release any carbohydrate Neither were glycosylglycerol groups isolated from any of the hydrolysates. These results strongly suggest that glycosidic substituents to a polyol phosphate chain are unlikely If such structures were present, formation of glycosylglycerol units should be favoured in HF (3) The results are also in agreement with the serological observations (7 8) that poly P did not cross-react with teichoic acids having the same constituents, but with the sugar in a glycosyl substitution. Most probably therefore, poly P is composed of repeating units of alternating glycerol phosphate, and sugar in one chain.

Hydrofluoric acid degraded the polysaccharide to smaller fragments. Most fragments, however had molecular weights above 600 according to the results on gel filtration The results of digestion of HF hydrolysate with phosphomonoesterase indicated a low percentage of monoester but these are cleaved more rapidly than diesters by HF (3) Some free glycerol, a trace of glucose, but no free glucosamine (or glucosamine phosphates) were detected.

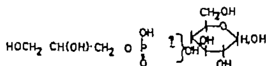


Fig 3 Tentative structure of S_I isolated from the hydrofluoric acid hydrolysate.

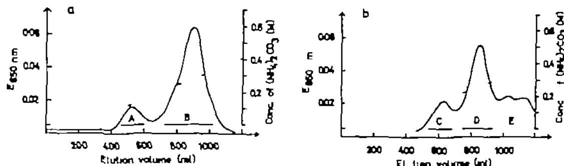


Fig. 1. DEAE-cellulose chromatography of hydrofluoric acid (a) and alkali (b) hydrolysates of poly P. The curves show the amount of phosphorus present.

phosphomonoesterase released some free glucose, glycerol and about 8 per cent of the phosphate. Similar treatment of the alkali hydrolysate released some free glycerol and about 15 per cent of total phosphate.

DEAE-Cellulose Chromatography of Hydrolysates

The elution patterns of HF and alkali degraded poly P (about 150 mg in each) from the DEAE-cellulose column are illustrated in Fig. 1 a and b respectively. Trace amounts of free glycerol were observed in the water eluates. The material of the HF hydrolysate was eluted in two peaks (A and B) concentrations of $(NH_4)_2CO_3$ being 0.11–0.16 and 0.21–0.33 respectively. The alkali-degraded material was eluted in three peaks (C, D, E), of which only two (C and D) eluted at 0.13–0.18 and 0.22–0.30 M $(NH_4)_2CO_3$ respectively were further analysed.

Gel Filtration of Hydrolysates

The gel filtration patterns of HF and alkali-degraded poly P on a column of Bio-Gel P-30 are shown in Fig. 2. The elution curves indicate that substances of different size were present. Apparently the HF hydrolysate contained a more uniform low molecular weight fraction than the alkali hydrolysate. On the other hand, the HF hydrolysate contained some non-degraded or slightly degraded material which was eluted just after the void volume. Further fractionation on a Bio-Gel P 2 column of the main peaks from the HF hydrolysate (23–28 ml) and the alkali hydrolysate (21–32 ml) showed that most of the material in both instances was eluted just after the void volume. A shoulder on the peak from alkali-degraded material indicated differences in size of the organic phosphates. Inorganic phosphate was in both instances eluted in late fractions, the content of this material being highest in the alkali-degraded material.

Chemical Analyses

The organic phosphates separated on DEAE-cellulose (A, B, C, and D) were further analysed by chromatography and fragments were isolated by preparative paper chromatography. Peak A seemed rather

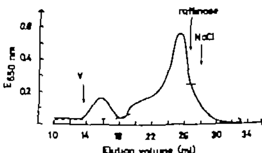


Fig. 2. Gel filtration of hydrofluoric acid (——) and alkali (– – –) degraded poly P. The curves are revealed by determination of phosphorus. V = elution volume of blue dextran.

Acta path. microbiol. scand. Sect. B 84 305–308 1976

IMMUNOCHEMICAL ANALYSIS OF AN UNUSUAL CELL WALL POLYSACCHARIDE FROM ANIMAL COAGULASE-POSITIVE STAPHYLOCOCCI

2 Probable Structure Based on Chemical and Serological Studies

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Endresen, C. & Grov A. Immunochemical analysis of an unusual cell wall polysaccharide from animal coagulase-positive staphylococci. 2. Probable structure based on chemical and serological studies. Acta path. microbiol. scand. Sect. B, 84 305–308, 1976

The teichoic acid of polysaccharide P (poly P) contains glycerol, glucose and phosphate. Hydrofluoric acid and alkali hydrolysates contain glycerol 1-phosphate, glycerol diphosphate and glucose 1-phosphate, but no glucosyl-glycerol fragments. Glucose and the serological activity of poly P were destroyed by periodate oxidation. Interaction with concanavalin A showed that the glucose is in α-configuration and that the hydroxyl groups at positions 3 4 and 6 are unsubstituted. Most probably the poly P teichoic acid is a polymer containing a repeating unit in which glycerol 1-phosphate is attached to the 2-position on α-D-glucose 1-phosphate

Key words Immunochemistry polysaccharide P animal staphylococci.

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Polysaccharide P (poly P) isolated from canine coagulase-positive staphylococci (5) was found to have an unusual structure It contained glycerol, glucose, glucosamine muramic acid, phosphate and the usual peptidoglycan amino acids (5) but no serological cross-reaction was obtained with standard teichoic acids, including poly Aα and Aβ poly Bα and Bβ (13 14)

Enzymatic digests of poly P and products from hydrolyses in hydrofluoric acid and alkali are described in a previous paper (4 All experiments indicated that no gly[illegible] substituents to the teichoic acid backbone were present. Furthermore the analysis of isolated fragments suggested that poly P is a polymer of a repeating unit of alternating glycerol phosphate and glucose and that the glucosamine present in the polysa[illegible] preparation belonged entirely to th[illegible] peptidoglycan moiety

This paper describes a close[illegible] examination of poly P and isolat[illegible]

MATERIALS AND [illegible]THODS

Polysaccharides and Standard [illegible]

Poly P was isolated from [illegible] aureus strain Z [illegible] poly Aα and Aβ (α- and β-N-acet[illegible]yl [illegible] teichoic acid) from [illegible] poly Bα and Bβ ([illegible] β-glucosylglycerol teichoic acids)

from *S. epidermidis* as described in (6) and (10) respectively. A crude poly I_2 from *S. lactis* I_{2}, a polymer containing a repeating unit in which D-glycerol 1-phosphate is attached to the 4-position on N-acetylglucosamine 1-phosphate (1) was extracted from whole bacteria by stirring with 10 per cent trichloroacetic acid at 4° C. After centrifugation (20 000 × g for 10 min) the supernatant was mixed with ethanol (2 vol.) and acetone (2 vol.). The crude polysaccharide, precipitated at 4° C, was isolated by centrifugation and used without further purification. Standard D-glucose, D-galactose, and D-glucosamine were obtained from Eastman (Rochester, N.Y., USA). A glycerophosphorylglucose fragment was isolated as described in (4).

Analytical Methods

Deamination. Samples of polysaccharide (5 mg) were added to 0.2 N HCl (0.5 ml) containing $NaNO_2$ (25 mg) and incubated at 20° C for 2 h. The solutions were then neutralised with N OH and dialysed against distilled water (15).

Periodate oxidation, hydrolysis in acid and alkali, *enzymatic dephosphorylation* and *chromatographic characterization* of products were as described in (4, 5). Testing of polysaccharides for reactivity with concanavalin A (con A) was carried out as before (5).

Serological Methods

Immune sera against the staphylococcal strains were produced in rabbits by intravenous injection of formalin-killed bacteria (12). *Double diffusion* analysis was performed as described in (7) and *immunoelectrophoresis* using an LKB apparatus, as described by the manufacturer (LKB Produkter, Sweden). The electrophoresis was run for 45 min.

Quantitative precipitation was carried out essentially as described in (8). The mixtures of antiserum (0.1 ml) and polysaccharide were incubated at 37° C for 30 min and then at 4° C overnight. The precipitates were spun down, washed twice in cold saline, dissolved in 0.2 ml of 0.1 N NaOH, and the protein concentration determined (11). The same procedure was followed in determining the precipitin curves between polysaccharides and con A (200 µl of 2 mg per ml). In *inhibition of the precipitin reaction* (8) glycerophosphorylglucose was used in concentrations up to 5 µmole, D-glucose and D-glucosamine in concentrations up to 100 µmole. The serum sample was adjusted to contain approximately 100 µg antibody protein, and the equivalent amount of polysaccharide was calculated from the quantitative precipitin curve.

RESULTS

Double Diffusion Analysis

Both intact and deaminated poly P gave a reaction of identity in agar diffusion against antiserum to strain Z_{11}. Deamination of glucosamine gives 2,5-anhydromannose (1) and further reduction by $NaBH_4$ produces 2,5-anhydromannitol. This substance was identified by gaschromatography. The gaschromatographic analysis also showed that all glucosamine was deaminated. Apparently no splitting of the polysaccharide (teichoic acid) occurred, since the serological activity was unchanged after dialysis. In immunoelectrophoresis, intact and deaminated poly P moved identically. Both preparations also reacted with con A, producing a line in agar. No serological cross-reaction was obtained be-

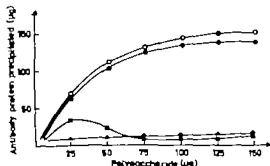


Fig. 1. Precipitation of poly P (–○–○–), deaminated poly P (–●–●–), poly Bα (–▲–▲–) and poly I_2 (–■–■–) by antiserum Z (0.1 ml).

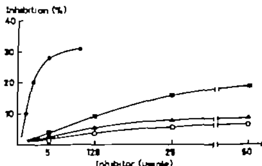


Fig. 2. Inhibition of the precipitin reaction between poly P (50 µg) and antiserum Z_{11} (0.1 ml) by D-glucose (–■–■–), D-galactose (–▲–▲–), D-glucosamine (–○–○–) and glycerophosphorylglucose (–●–●–).

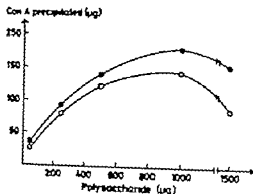


Fig 3 Precipitation of poly P (-○-○-) and poly Bα (-●-●-) by concanavalin A (con A) (400 µg)

tween poly P and any of the polysaccharide standards. Periodate oxidation destroyed not only the serological activity of poly P but also the reactivity for con A, and examination of the oxidized material showed complete absence of glucose.

Quantitative Precipitation

The amount of antibody precipitated by intact and by deaminated poly P seemed to be of the same order (Fig 1) whereas neither poly I nor poly Bα or Bβ showed significant precipitation in anti Z_1 serum.

D-glucose showed a small inhibitory effect on precipitation between poly P (50 µg) and homologous antiserum (0 l ml) (Fig. 2) The effect of D-galactose and D-glucosamine was negligible. Poly Bα in concentration up to 1 mg had an inhibitory effect of less than 5 per cent. However about 5 µmole of the glycerophosphorylglucose fragment inhibited the precipitation approximately 50 per cent

The precipitin curves between con A and poly Bα and poly P are illustrated in Fig. 3

DISCUSSION

The serological activity and specificity the reactivity for con A, and the electrophoretic mobility of poly P were the same whether glucosamine was present or not. This is in accordance with the earlier suggestion that glucosamine is a part of peptidoglycan fragments and not included in the poly P structure (4)

According to the result of periodate oxidation, glucose must be involved in the antigenic determinant(s) The lack of terminal glucosyl substitution (4) is in agreement with the lack of serological cross-reaction with poly Bα or Bβ and indicates glucose to be an integral part of the teichoic acid backbone. In most instances, teichoic acids have terminal determinants and specificities dependent on the carbohydrate residues attached to the glycerol- or ribitol-phosphate backbone. However evidence for non terminal carbohydrate determinants has been obtained with teichoic acids containing such residues integrated in their backbone (9)

The interaction with con A indicates that poly P contains α D-glucosyl substituents in which hydroxyl groups at positions 3 4 and 6 are unsubstituted (3) and compared to poly Bα only a 10–15 per cent smaller amount of precipitate is formed with con A on a weight basis.

The isolated glycerophosphorylglucose fragment consisting of nearly equimolar proportions of glycerol, phosphate and glucose (4) was shown to be an effective inhibitor of the precipitation between poly P and homologous serum. The results point to the conclusion that the majority of poly P preparations consists of chains of alternating glucose and glycerol linked through phosphodiester linkages (Fig. 4) The 1 position of both glycerol and glucose is occupied by phosphate (4) Thus, regarding glucose, no sorbitol was released by treatment of poly P with $NaBH_4$

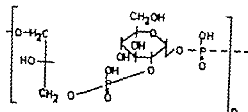


Fig 4 Probable structural unit of the poly P teichoic acid.

Apparently the 3-position of glycerol is also involved in a phosphoesterlinkage. Most of the products of both acid and alkali hydrolysates gave a rapid purple colour with the periodate-Schiff reagent (4) strongly indicating a free hydroxyl group at the 2-position of glycerol (2). With regard to glucose, the position of the second phosphoester linkage is more uncertain. However the reaction with con A precludes a linkage of phosphate to the 4-position as in poly I_1 a linkage at the 2-position being more probable. Most likely the poly P teichoic acid possesses a structure as shown in Fig 4 a polymer containing a repeating unit in which glycerol 1 phosphate is attached to the 2 position on α D-glucose 1-phosphate. This structure has a sterically accessible glucose residue with the free hydroxyl groups at positions 3 4 and 6 required for interaction with con A.

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PROTEOLYTIC DEGRADATION OF STAPHYLOCOCCAL α-TOXIN

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Dalen, A. B. Proteolytic degradation of staphylococcal α-toxin. Acta path. microbiol. scand. Sect. B, 84 309–314 1976.

Staphylococcal α-toxin of mol.wt. 39,000 was degraded at an alkaline pH by staphylococcal extracellular proteases resulting in the formation of three relatively stable intermediates with mol.wt. 27,500, 23,500 and 12,000. The intermediate with mol.wt. 27,500 which existed in two charged forms, was isolated by column chromatography and found to be non-haemolytic. Furthermore it could be obtained by proteolysis of α-toxin (mol.wt. 39,000) with chymotrypsin in low concentrations. This intermediate was further degraded by trypsin to the protein with mol.wt. 23,500 and 12,000

Key words: Staphylococcal α-toxin proteolytic degradation.

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There is general agreement about the heterogeneity of staphylococcal α toxin with regard to charge, whereas the estimates of molecular weights have varied from 15,000 to 45,000, even of toxins isolated from the same staphylococcal strain (11)

A simple procedure of purifying α-toxin involving heat precipitation at pH 5.0 and electrophoresis at pH 8.6, has recently been described (3) During this work, a peptide with mol.wt. 27,500 existing in two charged forms, was found to have antigenic relationship to a toxin with mol.wt. 39,000 Further studies on this relationship and the role of the proteolytic degradation of α-toxin are reported in this work.

MATERIAL AND METHODS

Staphylococcus aureus strain Wood 46, was propagated as described earlier (2) The medium based on Casamino acids (Difco) (vitamin-free) supplemented with an ethanol extract of yeast autolysate, and the incubation conditions, were as reported previously (2)

Protein Determination

This was performed by the Folin-Ciocalteu method (6) using crystalline bovine serum albumin as standard.

Double Diffusion Tests

Double diffusion in gel was carried out in Petri dishes containing a 3 mm thick layer of 1 per cent agarose (Behringwerke) The buffers used were Tris-chloride buffer (0.05 M, pH 7.5) phosphate buffer (0.05 M pH 7.0) and borate buffer (0.05 M, pH 8.6) The pattern used was a 3 mm centre antiserum well and 6 peripheral antigen wells of the same size at distance of 7 mm from the centre well. The antiserum used was an international standard for staphylococcal anti toxin, 1 ml containing 20 International units, obtained from Statens Seruminstitut, Copenhagen (Batch no. 88)

Analytical Gel Electrophoresis

Polyacrylamide disc electrophoresis was performed in glass tubes (0.5 × 7.5 cm) in Shandon

apparatus (Shandon Scientific Co., Ltd., London) The same buffer 0.37 M tris-glycine, pH 9.5 with 0 1 per cent SDS, was used both in the gel and in the electrode vessels. Samples of 10 to 15 µl with 10 to 100 µg protein were supplemented with SDS to a concentration of 1 per cent and left at room temperature for at least 1 h. Urea, if present in the sample, was not removed prior to electrophoresis. Before electrophoresis, the samples were diluted to an SDS concentration of 0 1 to 0.2 per cent by adding tris-glycine buffer (pH 9.5 0.07 M) together with 10 per cent glycerol (v/v) and bromphenol blue (0.00? per cent) The separating gel has a pH of 8.9 and contained 15 per cent (w/v) acrylamide, and the electrophoretic runs were done as previously described (2)

Purification of α-toxin (2)

Bacteria were removed from fluid cultures by centrifugation and filtration on Millipore filters, pore size 0.45 µ. The pH was adjusted to 5.0 and the fluids were then heated to 60 C for 30 min in batches of 300 ml. The precipitates formed were collected by centrifugation and then washed twice with borate buffer (pH 8 6, 0.03 M) The precipitates were dissolved in the same buffer containing 8 M urea. Polyacrylamide gels, pH 8.9 containing 7 per cent acrylamide, were made in tubes 0.7 × 8.0 leaving an open space of 0.5 cm at the top. This chamber was filled with the dissolved material. The reservoir buffer was 0.15 M borate buffer pH 8.6 The samples were electrophoresed at a constant voltage of 50 V for 45 min with the anode at the lower end of the tubes. The main fraction of α-toxin was electrophoretically immobil at this pH, while the contaminating proteins moved from the chamber

Column Chromat graphy

Bio-Gel P 150 and P-200 (Bio-Rad Labs., Richmond, Calif USA) were prepared in borate buffer (0 03 M, pH 8 6) containing 8 M urea The Pharmacia column used, 45 × 2.5 cm, gave a gel volume of 175 ml. Separation was done at room temperature. A peristaltic pump (1200 Vario Perpex, LKB) was used and extinction at 280 nm was recorded with a Uvicord II (LKB) Urea was removed from fractions by dialysis overnight at +4 C. In experiments where the rapid removal of urea was essential, a filtering device usually employed for concentrating urinary proteins (Minicon B 15 Amicon Corp., Lexington, Mass., USA) was used. Aliquots of 0.5 ml were concentrated 5 × The buffer to be used (acetate pH 5.0 0.05 M, phosphate, pH 7 0 0 05 M or borate, pH 8.3 0 05 M) was added, and the concentration procedure was repeated until the original volume (0.5 ml) with a urea concentration of less than 0.5 M was obtained.

Molecular Weight Determinations

The following marker proteins were bought from Boehringer Mannheim, West-Germany bovine serum albumin, mol.wt. 67,000 bovine pancreatic chymotrypsinogen, mol.wt 25 000, horse myoglobin, mol.wt. 17,800 and horse heart cytochrome C, mol.wt. 12,400. Electrophoresis was done in 15 per cent polyacrylamide gels and in tubes 12 cm long, but otherwise as described under Analytical gel electrophoresis"

Chemicals

Crystalline α-chymotrypsin and crystalline trypsin were obtained from Novo Industri (Copenhagen, Denmark) The other chemicals used were analytical grade reagents obtained from various commercial sources.

RESULTS

The heat precipitates contained two major proteins with mol.wt. 39 000 and mol.wt. 27,500 together with several minor proteins (Fig 1 a) The relative amounts of the two major components varied, but the protein with mol.wt. 39 000 dominated in most prep-



Fig 1 SDS-polyacrylamide gel electrophoresis of
a) heat precipitate of supernatant from fluid culture (pH 5 0, 60° C for 30 min)
b) α-toxin (mol.wt. 39,000) purified by electrophoresis of heat precipitates t pH 8.6,
c) mixture of α-toxin (mol wt. 39,000) and the protein with mol.wt. 27,500 obtained by column chromatography
d) purified protein with mol.wt. 27,500 obtained by column chromatography

arations. Preparative electrophoresis at pH 8.6 usually produced the protein with mol.wt. 39,000 and it had the characteristics of α-toxin (Fig. 1 b). The second component was present after electrophoresis in a minority of the preparations.

Attempts to separate the two components by column chromatography (Sephadex G 200 and Bio-Gel 200) with buffers of low tonicity were complicated by the precipitation of proteins in the column. By column chromatography on Bio-Gel P 150 or P 200 with borate buffer (pH 8.3, 0.05 M) containing 8 M urea a haemolytic mixture of the two proteins and the protein with mol.wt. 27,500 in the pure form was obtained (Fig. 1 c, d). No haemolytic activity of the pure protein could be detected. The protein was not precipitated upon removal of urea by dialysis at pH 7.0 and 8.3. A high molecular weight component (mol.wt. 115,000) was formed when the dissolved protein was left at room temperature, presumably representing a tetramer of the protein.

Mixtures of the two proteins gave two precipitation lines on gel diffusion in agar both fusing with the single line formed by the protein with mol.wt. 39,000 produced by preparative electrophoresis (Fig 2). Pure protein with mol.wt. 27,500 produced a single and somewhat diffuse precipitation line, giving a reaction of partial identity with that of haemolytic α-toxin. When high concentrations of protein with mol.wt. 27,500 were used, a barely visible precipitation line crossing the α toxin line was observed with a spur of the toxin line indicating some antigenic individuality of both proteins.

The antigenic relationship between the two proteins could be explained by proteolytic degradation of α toxin to a non-haemolytic protein with mol.wt. 27,500. Evidence for proteolytic degradation of α toxin in complex mixtures of extracellular proteins sought by dissolving heat precipitates in 8 M urea and dialysing samples against buffers (acetate, phosphate and borate, 0.05 M of pH 5.0 7.0 and 8.3) overnight at room temperature. An examination of the material by polyacryl-

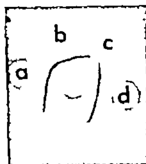


Fig 2 Double diffusion in gel showing antigenic relationship between α-toxin (mol.wt. 39 000) and protein with mol.wt. 27,500

a and d) mixtures of the two proteins (shown in Fig. 1 c)
b) purified α-toxin (shown in Fig. 1 b)
c) purified protein with mol.wt. 27,500 (shown in Fig. 1 d)

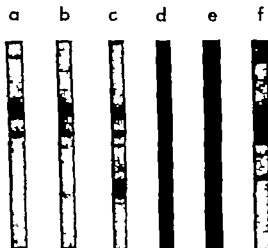


Fig 3 Proteolytic degradation of α-toxin demonstrated by SDS-polyacrylamide gel electrophoresis.
a) on dialysis of crude heat precipitates dissolved in 8 M urea at room temperature and pH 5.0,
b) on dialysis of crude heat precipitates dissolved in 8 M urea at room temperature and pH 7.0,
c) on dialysis of crude heat precipitates dissolved in 8 M urea at room temperature and pH 8.3
d) degradation of α-toxin (mol.wt. 39,000) to the protein of mol.wt. 27,500 by chymotrypsin (enzyme: protein ratio 1:10, 10 min incubation at 37 °C)
e) degradation of the protein with mol.wt. 27,500 by trypsin (trypsin added to d, enzyme: protein ratio 1:10, 10 min incubation at 37 °C)
f) as e but prolonging the incubation with trypsin to 20 min.

amide electrophoresis after dialysis showed the appearance of a number of peptides, with a molecular weight below 25,000 This was particularly evident if the precipitates formed during dialysis were studied (Fig 3 a, b c)

The proteolytic activity was highest at pH 8.3 but was also apparent at pH 5 0. The α toxin line was split into several closely spaced lines probably indicating a sequential loss of small peptides from α toxin. The relative amount of the protein with mol.wt. 27,500 increased at the alkaline pH with a corresponding reduction of α toxin. Two proteins with mol.wt. 23,500 and 12,000 accumulated increasingly with increasing pH.

The degradation of α toxin in complex mixtures was compared to that produced by low concentrations of chymotrypsin and trypsin. The α toxin, mol.wt. 39 000 was exposed to chymotrypsin and trypsin (10 μg trypsin or chymotrypsin to 100 μg α toxin) at pH 7.0 and 8.3 for 10 min at 37 C. The toxin resisted the action of trypsin under these conditions while chymotrypsin at pH 7 0 degraded the toxin to one main component with mol.wt 27,500 (Fig 3 d) The component with mol.wt. 27,500 was resistant to chymotrypsin on further incubation. Addition of trypsin to the chymotrypsin treated material gave a rapid degradation of the component with mol.wt. 27,500 to a protein with mol.wt. 23,500 and several low molecular weight peptides (Fig 3 e) By prolonging the incubation with trypsin to 20 min two peptides with mol.wt. 18,000 and 12,000 appeared (Fig 3 f)

A proteolytic activity of α-toxin resembling that of trypsin has been described (13) Mixtures of α toxin and the protein with mol.wt. 27,500 from Bio-Gel columns containing 8 M urea did not show evidence of proteolytic degradation when incubated at pH 5.0 7.0 and 8.3 at 37 C for 2 h. After the removal of urea by filtration on Minicon B 15 proteolytic degradation of the protein with mol.wt. 27,500 was evident after incubation under the same conditions. At pH 5 0 and even more at pH 7 0 a protein with mol.wt. 22,500 was formed. At pH 8.3 the protein with

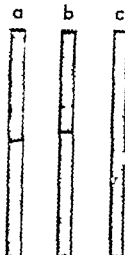


Fig 4 Proteolytic degradation of the protein with mol.wt. 27,500 in mixtures with α-toxin (mol.wt. 39 000) demonstrated by SDS-polyacrylamide gel electrophoresis. The mixtures were obtained by column chromatography and the composition is shown in Fig. 1 c Incubations were done at 37 C for 2 h at
a) pH 5.0 b) pH 7.0 c) pH 8.3

mol.wt. 27,500 was completely degraded to low molecular weight peptides (Fig 4 a, b c)

DISCUSSION

The presence of two charged forms of staphylococcal α-toxin, one major component with a pI reported between 8.4 and 8.6 and one minor component with a pI between 7.2 and 7 4 is supported by several studies (11) In addition an acidic component with a pI reported variously as between 5 0 and 6.2 and a basic component with a pI of 9 1 and 9.2 have been observed by using the isoelectric focusing method (2, 7 10) More divergent results have appeared concerning the N terminal amino acid of α toxin. *Coulter* (1) observed histidine and arginine, while *Wiseman & Caird* (12) found only histidine. In contrast to this, *Six & Harshman* (8, 9) observed alanine as the N terminal amino acid in both of their two differently charged forms. These variations and the differences in molecular weights would, as suggested by others, in-

dicate a modification of α toxin by proteolytic enzymes.

The lytic effect of α-toxin on various natural and artificial membranes is supported by a great number of studies. A trypsin-like effect of α-toxin which had been activated with trypsin was reported by *Wiseman* (11) and *Wiseman et al.* (13). However *Freer et al.* (4) could not confirm the evidence of a proteolytic mechanism in the mode of action of α-toxin.

From the present findings and from two previous studies (2, 3) it would seem that the primary form of α-toxin had a molecular weight of 39,000 and that it existed in two charged forms, the pI of the major component being 8.6 and the minor component 7.4. Conversion of the major to the minor component by the action of an arginase attacking intramolecular arginine (5) would be a reasonable explanation. Extracellular proteases modified the α toxin in various ways. Loss of low molecular peptides gave rise to several new species of toxins differing slightly in molecular weights. Through the action of a chymotrypsin-like enzyme a relatively stable intermediate with mol.wt. 27,500 was formed. The intermediate existed in two charged form (3) and was non-haemolytic. The lack of haemolytic activity might be related to the purification method used, since *Six & Harshman* (9) reported a haemolytic α toxin with the same molecular weight and the same pI (8.4) as the major component. The intermediate was sensitive to trypsin with the formation of a peptide with mol.wt. 23,500. This was further degraded to peptides with mol.wt. 18,000 and 12,000.

A similar degradation took place when the trypsin-sensitive intermediate with mol.wt. 27,500 was incubated with α toxin with mol.wt. 39,000. This finding supported the claims of a proteolytic activity of α-toxin (13). The presence of a contaminating protease could not be ruled out but seemed unlikely.

The pronounced tendency of α toxin and its derivatives to form high molecular complexes was evident in experiments with dialysis, column chromatography and isoelectric focusing. Optimal conditions for complex formation were pH 4.0 to 5.0 and heating up to 60 °C. At a pH below 3.5 complexes were dissolved with release of haemolytically active toxin. The presence of an acidic component of α-toxin with a pI of about 6.0 would seem to be adequately explained by acidic dissociation of toxin-containing complexes (2).

The α toxin component with a pI of 9.1 to 9.2 which appears by using the isoelectric focusing method could be accounted for by protein-protein interactions. α Toxin with mol.wt. 39,000, the stable intermediate with mol.wt. 27,500 and two basic proteins with mol.wt. 18,000 (pI 9.5) and 12,000 (pI > 11.5) were all found in this fraction (2). The two last peptides presumably originated from α-toxin through the trypsin-like degradation of the intermediate with mol.wt. 27,500.

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BRIEF REPORTS

THE EFFECT OF SPECIFIC ANTIBODIES ON THE INHIBITION OF LEUCOCYTE MIGRATION CAUSED BY STAPHYLOCOCCAL PEPTIDOGLYCAN

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Grov A. The effect of specific antibodies on the inhibition of leucocyte migration caused by staphylococcal peptidoglycan. Acta path. microbiol. scand. Sect. B, 84: 315–317, 1976.

Specific antibodies to the various antigenic determinants of staphylococcal peptidoglycan are tested for neutralization of the inhibiting effect of peptidoglycan on leucocyte migration. Antibodies to the C-terminal D-Ala-D-Ala group of pentapeptides and to the C-terminal of the glycine bridge showed high neutralizing effect, whereas that of antibodies to the tetrapeptide and to the glycan chain was negligible. The observed neutralization by antibodies against the outermost parts of peptide chains may be due to the inhibition of contact between peptidoglycan and cells.

Key words: Staphylococcal peptidoglycan; leucocyte migration; peptidoglycan antibodies.

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The inhibition of cell migration (MIR) is one of several biological properties elicited by bacterial peptidoglycans (8). Staphylococcal (1, 3, 7, 13) as well as streptococcal (1, 9, 10) peptidoglycans, and peritoneal cells from non-sensitized man, mouse and guinea pig (13), non-sensitized or sensitized guinea pig (1, 3, 7) and rat (9, 10) have been used to obtain MIR. It is supposed that the apparently non-specific effect is due to an intrinsic toxic factor of the peptidoglycans (10). In addition, fragments of staphylococcal peptidoglycan have been found to inhibit migration of guinea pig peritoneal exudate cells (3). Both N-acetyl amino sugars and peptide seemed necessary to obtain inhibition, the critical lower limit in molecular weight being approximately one thousand.

Peptidoglycan also possesses immunological properties (4), and the intention of the present study was to look for a possible neutralization or blocking of the inhibiting effect on cell migration by specific peptidoglycan antibodies.

Materials and Methods

Peptidoglycan

Peptidoglycan from *Staphylococcus aureus* strain Cowan I, was prepared and tested as described in (5). The fragment (tetrasaccharide-peptide, mol.wt. 2,000) used was that isolated and described before (3).

Specific Antibodies

Rabbit antisera to peptidoglycan of *S. aureus* Cowan I was prepared (6) and the specific antibodies anti-(Gly)5, anti-tetrapeptide (L-Ala-γ-D-Glu-L-Lys-D-Ala) and anti-D-Ala-D-Ala were isolated from the anti-peptidoglycan sera on immunosorbents of synthetic peptides linked to Sepharose (6). Anti-glycan antibodies were precipitated from the absorbed sera by 2 parts of saturated $(NH_4)_2SO_4$, the dissolved and dialysed materials being further purified on columns of DEAE-cellulose (11). IgG from non-immunized rabbit and from non-absorbed anti-peptidoglycan sera were also prepared in this way. The methods used for the estimation of protein concentrations and the equivalence zones between specific antibodies and peptidoglycan were those described before (2).

TABLE 1 *Migration Inhibition Test*

No. of cultures (capillary tubes in brackets)	Preparations added to the medium	µg/ml	Mean relative migration (%)
4 (32)	None	–	100
2 (16)	NR IgG	50	100
2 (16)	R. anti-Pg IgG	50	100
4 (32)	Pg	25	< 20
3 (24)	Pg/anti-Pg IgG	25	60
3 (24)	Pg/anti-(Gly)5	25	50
3 (24)	Pg/anti-L-Ala-γ-D-Glu-L-Lys-D-Ala	25	25
3 (24)	Pg/anti-D-Ala-D-Ala	25	75
3 (24)	Pg/anti-glycan	25*	< 20
1 (12)	Tetras.-pept.	25	75
1 (12)	Tetras. pept./anti-D-Ala-D-Ala	25*	100

NR IgG = normal rabbit IgG Pg = peptidoglycan, Tetras.-pept. = tetrasaccharide-peptide, mol.wt. 2 000 (3) * Amount of antigen.

Migration Inhibition Test

Samples of peptidoglycan mixed with equivalent amounts of specific antibodies were incubated with gentle stirring for 2 h at 37 C and then for 24 h at 4 C. The peptidoglycan-IgG complexes were isolated by centrifugation, washed once in cold saline, and suspended in supplemented Eagle's medium (7) to a concentration of 25 µg peptidoglycan per ml. The rest of the procedure was performed as earlier described (7) using peritoneal exudate cells from non-sensitized guinea pig.

Results and Discussion

The results of the migration inhibition test are summarized in Table 1. Rabbit IgG (50 µg per ml of supplemented Eagle's medium) from normal or anti-peptidoglycan sera did not influence the cell migration, whereas 25 µg of peptidoglycan per ml of medium gave complete inhibition.

Antibodies to the D-Ala-D-Ala determinant of free pentapeptides showed a neutralizing effect on the inhibitory property of peptidoglycan. Inhibition of cell migration was reduced to about 25 per cent (75 per cent migration). These antibodies completely neutralized the inhibiting effect of the *tetrasaccharide-peptide fragments. Similarly* a somewhat slighter effect was observed using antibodies to the penta-glycine bridges with a free C-terminus. Antibodies to the other determinants seemed to be of minor importance in this context. Complete blocking of the inhibiting effect of peptidoglycan was not obtained either with IgG of anti-peptidoglycan whole serum (60 per cent cell migration) or with a mixture of specific antibodies. The amounts of antibodies used were equivalent to the amount of peptidoglycan. It may be that the complex peptidoglycan preparations were insufficiently homogenised and that the antigenic determinants were not completely blocked by antibodies. The inhibiting effect of the relatively simple tetrasaccharide-peptide fragment was completely neutralized by anti-D-Ala-D-Ala antibodies.

A slight decrease in migration inhibition by an excess of precipitating antibodies to streptococcal peptidoglycan is reported (10). The response to the various antigenic determinants *is found to be* variable (4) and the concentration ratio between antibodies of different specificities vary from one serum to another. The immunodominant site of peptidoglycan from Streptococcus group A variant has, however, been found to be the COOH-terminal D-Ala-D-Ala (12).

Apparently antibodies to peptidoglycan affect the capacity of the peptidoglycan to inhibit cell migration. Only those antibodies directed against the outermost part of chains which are not cross-bound seem to be involved in neutralization. This may indicate that the mechanism is only an inhibition of a direct physical contact between peptidoglycan and cells, a contact which normally may become established via the peptide chains. It has been shown that neither peptides alone (1 3) nor oligosaccharides (3) were able to *inhibit cell* migration, which may indicate a dual mechanism. Oligosaccharides having peptide chains with free COOH-terminus did inhibit cell migration (3).

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ISOLATION OF *YERSINIA ENTEROCOLITICA* FROM DANISH SWINE AND DOGS

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Pedersen, K. B. Isolation of *Yersinia enterocolitic* from Danish swine and dogs. Acta path. microbiol. scand. Sect. B, *84:* 317–318, 1976.

Seventeen strains of *Yersinia enterocolitica* were isolated on examination of the caecal contents from 100 bacon pigs at slaughter In another material consisting of 222 pigs with various diseases, the bacterium was found in 12 cases. Three out of 40 dogs were positiv for the bacterium. Seven of the porcine and one of the canine strains belonged to serotype O 3 which is a human pathogen.

Key words: *Yersinia enterocolitica* isolation swine dogs.

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Human infections with *Yersinia enterocolitica* (Y.e.) have been reported with increasing frequency during the last decade (4) The distribution and ecology of the bacterium is incompletely known. However the fact that the serotypes that are pathogenic for man, i.e types 3 and 9 are frequently isolated also from pigs, has incriminated swine as a probable source of human infection The purpose of the present work was to examine t what extent Danish swine and dogs might be infected with Y.e.

Caecal or colonic contents from 222 swine and 40 dogs received at this instit te for routine diagnostic purposes from medio September 1975 to February 1976 were examined. Selection by post mortem diagnosis was not made. In addition, caecal contents were examined from 100 bacon pigs at slaughter (January 1976) The material from the diseased animals was cultured on MacConkey agar both directly and after enrichment Cultures were incubated both t room temperature and at 37° C for 24–48 hours. The slaughterhouse material was examined only after enrichment. Enrichment was performed in phosphate buffered saline (pH 7 6) at 4 C for three weeks. A 10 per cent suspension was used. Lactose-negative colonies were considered to be Y.e suspect and subjected to biochemical characterization by conventional tests Serological typing was performed by professor *S Winblad* Malmø Sweden

Y.e was isolated from 12 (5 4 per cent) of the

TABLE 1 *Serological and Biochemical Typing of* Y enterocolitica *Isolated from Swine and Dogs*

Material	No. examined	No. of Y.e positive samples (%)	O-serotype* 3	5	6	7	12	17	19	26b	26c	Untypable
			Biotype§ 4	1	1	1	3	1	1	1	1	1
Bacon pigs at slaughter	100	17 (17)	4	3	4	1	2	1	1	1		
Diseased pigs†	222	12‡ (5.4)	3	5	2	1					1	1
Diseased dogs†	40	3 (7.5)	1	1		1						

* Performed by prof *S Winblad* according to the principles described by *Winblad* (10)
§ According to *Niléhn* (5)
† Pigs and dogs with various diseases received for routine diagnostic examination.
‡ One of the samples was positive for two different serotypes.

pigs and from 3 (7.5 per cent) of the dogs received for diagnostic purposes (Table 1) All the isolations were made after enrichment. Ten of the strains were recovered from pigs in the age group 5–11 weeks, which constituted 84 per cent of the material. One of the pigs was positive for two different serotypes (3 and 5) Three strains were obtained from the 20 pigs more than 11 weeks old. The bacterium was not isolated from any of the 15 pigs less than 5 weeks old. Seventeen strains were isolated from the 100 bacon pigs. Table 1 also shows the serological and biochemical typing of the isolates. Seven of the porcine and one of the canine strains belonged to serotype 3

The eight strains of serotype 3 showed distinct β-haemolysis on bovine blood agar after storage of the culture at room temperature for 3–12 days An unsharp zone of haemolysis was seen in the two serotype 12 strains. None of the other serotypes were haemolytic.

From 3 of the herds from which Y.e. had been isolated from diseased pigs, 10–15 additional faeces samples were examined. Y.e. was not isolated from any of these samples

This preliminary study has shown that Y.e can be isolated both from pigs with various diseases and from apparently healthy pigs at slaughter There was no evidence to suggest a pathogenic role of this organism for swine. The results are comparable with those obtained in Canada (7) Germany (3) Holland (2) and Japan (8, 9) The recovery of serotype 3 from pigs might suggest that swine can act as a source of infection to man. The occurrence of Y.e. in dogs must, as also stressed by others (1 8) cause more apprehension because of the close contact of dogs with man. The human carrier rate seems to be low (6)

The author is grateful to professor *S Winblad* Malmo, Sweden, for serological typing and for valuable advice during the work. Thanks are also due to Mrs. *Minna Petersen* for the very skilful technical assistance.

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AGAROSE ELECTROPHORESIS COMBINED WITH SECOND DIMENSIONAL CETAVLON PRECIPITATION A NEW METHOD FOR DEMONSTRATION OF ACIDIC POLYSACCHARIDE K ANTIGENS

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Ørskov F Agarose electrophoresis combined with second dimensional Cetavlon precipitation. A new method for demonstration of acidic polysaccharide K antigens. Acta path. microbiol. scand. Sect. B, 84 319–320, 1976.

A new method was developed for detection of acidic polysaccharides in bacterial extracts of *E. coli* using agarose electrophoresis of the extracts combined with Cetavlon precipitation in the second dimension.

Key words Acidic polysaccharides, agarose electrophoresis, Cetavlon precipitation, *Escherichia coli*

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Recently *Ørskov et al.* (3 4 5 6, 9) have redefined the *Escherichia coli* K antigen definition recognizing primarily two main groups of antigens the polysaccharide K antigens, which are essentially thermostable and the protein K antigens, which are on the whole thermolabile.

The polysaccharide K antigens so far examined contained acidic groups and thus in immunoelectrophoresis they move towards the anode. These acidic K antigens are determined by chromosomal genes and two groups of antigens have been described one in which the specificity is determined at a locus called *kpsA* close to *serB* and another which has its main locus close to the histidine and the *rfb* locus (7 8) The last mentioned polysaccharide K antigens, K(A) are usually found together with O antigens O8, O9 O20 and O101 The relationship between the K(A) antigens and the polysaccharide side chains of the above listed O antigen lipopolysaccharides (9) is not yet fully understood.

K determination of unknown strains can be carried out by slide agglutination in the appropriate test sera, but due to the existence of several common surface antigens, most of them thermolabile, a method relying on gel precipitation will provide a more saf K determination.

Recently *Semjen et al.* (11) have described a counter-current immunoelectrophoresis technique for *E. coli* K determination which is now used in our laboratory As the number of strains that cannot be K determined by the present about 60 K test sera is high, the final number of *E. coli* K antigens is presumably much higher It is therefore a great practical problem to decide whether these K non-typable *E. coli* have an acidic polysaccharide or not, since the only method available at present is to produce an OK serum with the strain and test this in immunoelectrophoresis (4 5 6)

We have therefore searched for substance which would react with acidic polysaccharides after agar electrophoresis. After many trials with different stains we observed that Cetavlon, which is wellknow for its reaction with acidic polysaccharides (10) when used instead of antiserum gave neat precipitation arcs with negatively charged polysaccharide antigen in immunoelectrophoresis"

The antigens used are those found in the two types of extracts described previously (5 6) One

of these is the supernatant of a bacterial suspension from broth agar plates, 10^{9} to 10^{11} bacteria/ml, heated at 60 C for 20 min, while the other type consists of the 60 C extract heat treated further at 100 C for 1 hour. A third type of extract is a similar suspension heated at 100 C for 1 hour which is used directly.

The electrophoresis is carried out in agarose for 90 min at a voltage of 7 V/cm with barbiturate buffer pH 8.6 as described previously (6). After electrophoresis is completed, the trough is filled with an 0.2 per cent solution of Cetavlon® (hexadecyltrimethylammonium bromide). The electrophoresis slides are incubated in humid atmosphere at room temperature. Often the precipitates can be observed after a few hours, but as a routine the results are read again after 20 hours. The precipitates can be stained by Alcian blue, preferably using the method of *Crowle* (1). The best stained preparations have been obtained by direct staining of the slides without previous washing or drying. If drying has to be carried out it should be remembered that direct drying of the slides without previous washing in distilled water will cause the precipitates to redissolve due to increases in molarity of salts in the agarose during the drying process.

We have examined all *E. coli* O and K antigen test strains and many other strains and our expectations have been fully confirmed, i.e. acidic K polysaccharides show precipitations at the expected location according to the immunoelectropherogram: 1) The K(A) antigens most often do not move far away from the application well, 2) the remaining polysaccharide K antigens—not K(A)—move farther away from the well, 3) the lipopolysaccharides containing acidic polysaccharide side chains precipitate close to the well on the anodic side like the K(A) antigens and thus cannot easily be distinguished from these by this test. However, hitherto the K(A) antigens have only been found in association with few well-defined O antigens (O8, O9, O20 and O101) and since O determination will always precede the K determination, this ambiguity is no real problem. Strains having both an acidic lipopolysaccharide and an acidic polysaccharide K antigen will, as anticipated, show two Cetavlon precipitation arcs, one farther away from the application well than the other.

Unexplained Cetavlon precipitation arcs were never observed with the *60 C* and the *60 to 100 C* extracts. When using the "rapid" extract where the bacteria are heated at 100° C, we have several times, due to a fast moving component, observed a line close to the anodic end of the trough in addition to the above-mentioned precipitation lines. Most likely this is caused by nucleic acids released from the cells by boiling; this line might be misinterpreted as a K line.

Up to now it has been the accepted procedure to rely mainly on the agglutinability or inagglutinability of the live strain in the homologous O serum in order to decide whether or not a strain contains a K antigen (2). However, according to our experience the so-called O inagglutinability which does support the existence of a K antigen, is too erratic a phenomenon to be used for any definite decision.

The Cetavlon electrophoresis technique is especially useful for examination of those frequently occurring strains which produce an acidic polysaccharide K antigen but are not inagglutinable in O serum.

The technique of agar electrophoresis combined with Cetavlon precipitation is of great help when it has to be decided whether or not to proceed with the serological analysis of a possible polysaccharide K antigen. Therefore this technique should be used in preference to the O inagglutinability test to decide if an *Enterobacteriaceae* strain has a polysaccharide K antigen. Experiments are in progress to develop this technique also for a quantitative measurement of polysaccharide K antigens.

Our thanks are due to Mrs. *Anette Bolsen* for excellent technical assistance.

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FIVE NEW *ESCHERICHIA COLI* K ANTIGENS, K95 K96, K97, K98 AND K100

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Ørskov I & Ørskov F Five new *Escherichia coli* K antigens, K95 K96, K97 K98 and K100
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Five *Escherichia coli* strains were established as antigenic test strains for five new polysaccharide K antigens K95, K96 K97 K98 and K100 K95 to K98 served already as test strains of O antigens O75 O77 O81 and O107 respectively F147 which is test strain of K100, had O antigen O75.

Key words *Escherichia coli* K antigens fiv new antigens.

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In a previous investigation (5) all E coli O antigen test strains were examined by immunoelectrophoresis (IE) in homologous O and OK antisera. A special polysaccharide K antigen could be demonstrated only in a limited number of the strains and most of these were strains with already numbered K antigens. About 95 of the strains were hitherto labelled K? because no K antigen *examination had ever been undertaken. In* 9 of these strains (O19 O75 O77 O81 O103 O107 O116, O117 O120) an anodic K antigen line was demonstrated. It was later decided to give four of these K antigens an official status as K95 K96 K97 and K98. The present paper reports the examination of these and of the K antigen of strain F147 which has already been published as K100 (2) Two *E. coli O75.H5 strains*, called Easter and 89 had been found to cross-react with the capsular polysaccharide of *H in fluenzae type b (1) The* K *antigen* of the *E. coli* strains was identical with that of an O75.H5 strain, F147 isolated in 1950 from cases of infantile diarrhoea in Rostock, DDR.

MATERIALS AND METHODS

Strains

The following six strains are those with new K antigens

Original number	O.H serotype	New K antigen
E3b	O75.H5	K95
E10	O77.H⁻	K96
H5	O81.H	K97
H705	O107 H27	K98
35w	O117 H4	K98
F147	O75 H5	K100

Three f rther strains were particularly involved in cross-reaction studies. These were test strains of O120 = 35w = O120 K H6, of K51 = Su3973/

41 = O8:K31(A):H and of O116 = 28w = O116 K H10.

Media. Beef broth plates (7) were used when cultures were tested by agglutination technique, while the D5 medium (8) containing 0.03 per cent glucose (9) was employed for preparation of extracts.

Methods

Antiserum production, agglutination technique and procedures for absorption of antisera were performed as described by *Kauffmann* (5). Antisera were used undiluted, whether absorbed or not, for precipitation tests but diluted 1.5 or 1 10 for the agglutination test. Precipitation by double diffusion in gel was carried out with filter paper discs (10). The immunoelectrophoretic technique (8) and the preparation of extracts (4) have been described previously.

The strains known from IE tests to have new unnumbered acidic polysaccharide K antigens (O49 O75 O77 O81 O103 O107 O116 O117 O120) were examined for cross-reactions in slide agglutination with all OK antisera, i.e. those of all established K antigens and of these possibly new types. Positive cases were then examined in the gel diffusion test and, if the 100 C extract was positive in that test, further examination was carried out.

RESULTS

Two of the strains reported here, i.e. test n of antigen O75 (from now on referred as strain O75) and F147 have antigen O75 but different K antigens (Fig 1 1 and 2). Their pattern in IE is of type 1 Aa (4) meaning the O antigen precipitation line on the cathodic side of the antigen well and the K antigen moving towards the anode. The K antigen of F147 reported as antigen K100 (2) cross-reacts with K antigen of strain O120 but they are not identical. The reaction in OK antiserum O120 is shown in Fig 1 5. Relationship to K antigens of K18 and K22, which are mutually closely related, is also found, though not regularly. Strain O120 belonging to IE group 2a (4) (O and K antigens on the anodic side of the the K antigen moving further toward Fig 1 3 and 4) cross-reacts of K31 (IE group 1 Ba (4) me gen on the cathodic side of the though often not ble, a

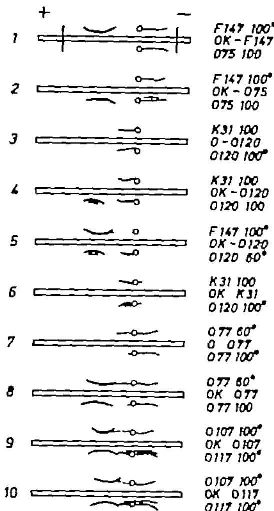


Fig 1 1–10 Immunoelectropherograms. The troughs contain OK or O antisera prepared against non-heated formalinised culture or 100° C culture respectively. The wells contain 60° C or 100 C extracts. 60° C extract is supernatant from suspension of a plate culture heated at 60 C for 20 minutes. 100 C extract is the 60° C extract heated t 100 C for 1 hour

on the anodic side of the well, Fig. 1 6). This cross-reaction indicates a relationship between the determinant group of the acidic lipopolysacc O120 and the acidic K antigen pol of K31 (Fig 1 4 and 6) on of OK antisera with b ture of strains O75 and In both cases agglu d culture Simi-

TABLE 1 *Tube Agglutination Tests with the Two Strains Having Antigen O75*

Antigens	Antisera				
	O	OK-O75		OK-F147	
	O75	Unabs.	Abs. with homol. 100 C	Unabs.	Abs. with homol. 100 C
Non-heated:					
O75	640	10240	2560	80	0
F147*	n.d.	320	0	320	80
100° C.					
O75‡	40960	20480	0	10240	0
F147‡	n.d.	20480	0	5120	0

0 = titre < 20.
40960 = titre ≧ 40960.
n.d. = not done.
OK F147 antiserum contains H5 antibodies, but this fact was not considered of importance here.
Living suspension from broth agar plate, formalinized.
‡ Suspension from broth agar plate, heated at 100 C for 1 hour

larly IE as well as double diffusion in gel showed that K precipitins remained after the absorption, particularly in the case of strain O75 The K antigen of this strain was numbered K95 The O75 strain agglutinated in many OK antisera on the slide, but no significant cross-reaction to other polysaccharide K antigens was found in the gel diffusion test.

The IE pattern of test strains O77 and O81 in homologous O and OK antisera is of type 1 Aa (4) That of O77 is shown in Fig. 1 7 and 8. Table 2 shows the result of agglutination with both test strains. In these cases no agglutinins remained after absorption with homologous, boiled culture. No reaction in other OK antisera was seen in the slide agglutination test. The K antigens of O77 and O81 were established as K96 and K97 respectively

Test strains O107 and O117, both of IE group 1 Aa, are known to have related O antigens. Examination in IE revealed a cross-reaction also among their K antigens (Fig 1 9 and 10) From results of agglutination tests in unabsorbed and cross-absorbed antisera no conclusion could be drawn as to the degree of relationship (Table 3) From gel diffusion tests, some of which are shown in Fig 2, it was concluded however that the K antigens of the two strains are identical. In addition to

TABLE 2. *Tube Agglutination Tests of Non-Heated and Heated Cult r in Homologous O and OK Antiser of O77 and O81*

Antigen		O antiserum	OK antiserum	
			Unabs.	Abs. with homol. 100° C
O77	Non-heated	0	80	0
	100° C	2560	1280	0
O81	Non-heated	640	160	0
	100° C	5120	1280	0

See key to Table 1

n

TABLE 3 *Tube Agglutination Tests with the Two Strains Having Related O Antigens O107 and O117*

Strains	Antisera							
	O	OK-O107			O	OK-O117		
			Abs. with				Abs. with	
	O107	Unabs.	hom. 100 C	O117 100° C	O117	Unabs.	hom. 100° C	O107 100° C
Non-heated:								
O107	0	320	0	40	160	640	0	40
O117	0	320	0	0	80	2560	20	160
100 C:								
O107	1280	2560	0	320	2560	2560	0	0
O117	640	2560	0	0	2560	2560	0	320

See key to Table 1
OK-O117 contains H4 antibodies, a fact which is not considered of importance here.

absorption with O107 and O117 100 C cultures (Fig. 2 a, b, c) the OK antiserum O117 was absorbed with 100 C culture of test strain of antigen O116. This was done because O116 and O117 cross-react in the slide agglutination test. In the gel diffusion test a cross-reaction is also demonstrable, but the lines are weak and difficult to attribute to either an O or a K antigen reaction. In Fig 2 d, it is seen that the K line of O117 is undisturbed by the absorption with the O116 strain The K antigens of O107 and O117 is established as K98. Strain O107 was chosen as test strain of this antigen because it did not react on the slide in other OK antisera, whereas strain O117 agglutinated on the slide in several OK antisera. None of these reactions were confirmed with 100 C extracts in gel diffusion test, except the doubtful reaction in OK antiserum O116.

The K antigens of the remaining K antigen containing O test strains (O49 O103 O116, O120) found previously are not given official K antigen numbers, at least not yet. This is mainly due to the fact that the antigens, although undoubtedly present, give too irregular reactions. It suffices to say that the K antigen of O120 is related to K100 as already mentioned.

DISCUSSION

The five polysaccharide surface antigens established in the present paper are all designated as K antigens without any further classification as either L or B antigens. The difference between L and B antigens was formerly based on the principle that the antibody binding capacity of the L antigen apparently was destroyed after heating at 100 C, while this was not the case with the B antigen both caused inagglutinability of untreated culture in O antiserum (3) By reexamining three non motile L antigen test strains, K12, K51 and K52, we showed previously that the K titre of K51 and K52 was lowered by absorption with homologous, boiled culture and practically eliminated when undiluted OK antiserum was absorbed. In the gel diffusion test, where only undiluted antiserum could be used, the absorption caused depletion of K antibodies in all three OK antisera. It was concluded that the three classical L antigens could not be differentiated from B antigens (6) with regard to agglutinin binding capacity

To-day we therefore disregard the distinction between L and B antigens and just denote them as K antigens. For establishment of new polysaccharide K antigens the presence of a marked K line in an immunodiffusion test carried out with a 100 C

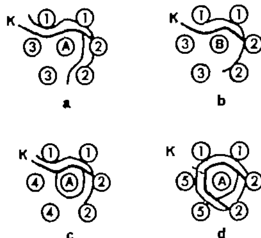


Fig 2 a, b c d. Gel precipitation tests with 100° C extracts of O107 and O117 in OK-O117 antiserum unabsorbed and absorbed.
A = extract of O117
B = extract of O107
1 = OK antiserum O117
2 = O antiserum O117
3 = OK antiserum O117 absorbed with O117 100° C
4 = OK antiserum O117 absorbed with O107 100° C
5 = OK antiserum O117 absorbed with O116 100° C
K = K antigen line

extract is required, while the K agglutinin binding capacity as well as the badly defined phenomenon of O inagglutinability are left out of consideration as regards presence or non-presence of a polysaccharide K antigen. Against this it could be argued that a few K antigens are very bad immunogens—a fact which might obscure their presence. This is true but we consider it less harmful to describe a strain with a weakly developed K antigen as K than to establish K antigens which are non-existing.

Cross-reactions between acidic lipopolysaccharides and K antigen polysaccharides as found here between O120 and K31 will be dealt with in a later paper

Our thanks are due to Mrs. *Marianne Hansen* for excellent technical assistance.

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A SIMPLE PROCEDURE FOR THE PURIFICATION OF STAPHYLOCOCCAL α-TOXIN

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Dalen, A. B. A simple procedure for the purification of staphylococcal α-toxin. Acta path. microbiol. scand. Sect. B, 84 326–332, 1976.

Staphylococcal α-toxin was produced in a fluid medium based on acid hydrolysed casein using strain Wood 46 α-Toxin and several other proteins were precipitated from bacteria-free culture supernatants by heating at 60° C for 20 min. The process was influenced by the pH of the solution. The toxin was completely inactivated and the precipitate contained a number of proteins if the pH of the solution was adjusted to 4.0–5 0 Heat precipitation of solutions having a pH of 6.0–7.0 resulted in a partial inactivation of α-toxin. The precipitates at this pH contained less of the additional proteins and had higher relative amounts of α-toxin than precipitates formed at a lower pH. The precipitate was dissolved in 8 M urea with the resultant activation of the haemolysin. Pure α-toxin with a molecular weight of 39,000 was obtained by electrophoresis in 8 M urea at pH 8.6 in ordinary tubes for polyacrylamide electrophoresis. The separation time was 45 min. The minor component of α-toxin with a pI of 7 4 could be demonstrated by the same method. A non-haemolytic protein with a molecular weight of 27,500 which existed in at least two charged forms, was shown to ha e an antigenic relationship to the toxin with a molecular weight of 39 000.

Key words Separation staphylococcal α-toxin.

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Staphylococcal α toxin has been highly purified in a number of laboratories in recent years, but no generally accepted purification method has yet been adopted (1) The purified toxin has been heterogeneous in charge (3) and molecular weights between 21,000 and 50,000 have been reported (1 12) Extracellular proteins from fluid cultures have been concentrated by precipitation with methanol, ammonium sulphate (1) and trichloroacetic acid (5) or by evaporation (10) Subsequent purification has been achieved by ion-exchange and by various electrophoretic means (1)

α Toxin readily undergoes spontaneous aggregation to high molecular weight polymers of low biological activity This process is enhanced by heating to 60–70 C, which results in the formation of insoluble, non-haemolytic precipitates. Haemolytic activity is restored to such precipitates by treatment with 8 M urea (2) at low pH or by heating to 90–100 C (5)

The use of heat in the concentration of α toxin from fluid cultures and the subsequent

purification by electrophoresis at the isoelectric point are described in this work.

MATERIALS AND METHODS

Bacterial Strain

Staphylococcus aureus, strain Wood 46, phage type 42 D (1000 RTD) which has been cultured for several years in this laboratory was used. It was grown on human blood agar and plated out on rabbit blood agar every two months, those colonies which showed wide zones of haemolysis being chosen for further propagation.

Prod ction of α-Toxin

The medium contained (per litre): Casamino acids (vitamin-free) (Difco Laboratories, Detroit, Mich USA) 20 g thiamine hydrochloride, 0.1 mg; nicotinic acid, 6 0 mg glycerol, 0.5 per cent (v/v) K_2HPO_4, 1.0 g. The medium was supplemented with an evaporate of 50 per cent (v/) ethanolic extract of 10 g yeast autolysate (Oxoid) per litre medium. The pH was adjusted to 7 1 with 5 N NaOH, and the medium was sterilized by Millipore filtration, pore size 0.45 µ. Incubation was performed on a rotary shaker (Controlled Environment, Incubator Shaker New Brunswick Scientific, New Brunswick, N J USA) 150 strokes/min at 37 C. A starter culture of 100 ml was grown overnight (15–18 h) and 10 ml of this was used to inoculate larger cultures distributed in 300 ml aliquots in 2 litre flasks. After overnight incubation, bacteria were removed by centrifugation and filtration on Millipore filtres, pore size 0.45 µ

Haemolytic Assay

Since the inactivation of haemolysin on dilution is a problem, a two-fold dilution in borate buffer (0.03 M, pH 8.3) containing 8 M urea was done. Aliquots of 20 µl from each dilution step were added to 1 ml of a 1 per cent rabbit erythrocyte suspension in phosphate buffered saline. The tubes were incubated at 37 C for 30 min and then read visually one haemolytic unit being defined as the amount lysing 50 per cent of the test suspension.

Protein Determination

This was performed by the Folin-Ciocalteu method (7) using crystalline bovine serum albumin as standard.

Do b Diffusion T st

Gel diffusion was carried out in petri-dishes containing a 3 mm thick layer of 1 per cent agarose (Behringwerke) in a trischloride buffer (0.05 M, pH 7.5) with a 3 mm centre antiserum well and 6 peripheral antigen wells of the same size at 7 mm distance from the centre well. The antiserum used was an international standard for staphylococcal anti-toxin, 1 ml containing 20 I.U., obtained from Statens Seruminstitut, Copenhagen (batch no. 88)

Analytical Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in glass tubes (0.5 × 7.5 cm) in a Shandon apparatus (Shandon Scientific Company Ltd., London) The separating gel of pH 8.9 contained 15 per cent (w/v) acrylamide (The British Drug Houses Ltd., Poole, England) Gels were polymerized with ammonium persulphate and N,N'-N' tetramethylene diethylenediamine. The same buffer (0.37 M trisglycine, pH 9.5) was used both in the gel and in the electrode vessels (5) When sodium dodecyl sulphate (SDS) was employed, its concentration in the gels and in the reservoir buffers was 0.1 per cent (w/) Samples of 10–15 µl containing 20–100 µg of protein, were supplemented with SDS to a concentration of 1 per cent and left at room temperature for at least 1 h. Urea, when present in the sample, was not removed. Before electrophoresis the samples were dil ted to an SDS concentration of 0.1 to 0.2 per cent by adding trisglycine buffer (pH 9.5 0.07 M) together with 10 per cent glycerol (v/v) and bromphenol blue (0.002 per cent) The samples were layered on top of the gels, stacking was done with constant voltage of 20 V and then increased to 100 V until the tracking dye had reached the lower anodic end of the tube. The gels were stained with Coomasie Brilliant Blue R 250 (4)

Preparative Electrophoresis

Heat precipitates were washed twice in borate buffer pH 8.6, 0.03 M The precipitate was then dissol ed in 0.03 M borate buffer (pH 8.6) containing 8 M urea. Polyacrylamide gels, pH 8.9 containing 7 per cent acrylamide were made in tubes 0.7 × 8.0 cm, leaving an open space of 0.5 cm at the top. This chamber was filled with heat precipitated proteins dissolved in urea. The reservoirs contained 0.15 M borate buffer pH 8 6. Similar gels were made with phosphate buffer (0.15 M, pH 7 4) The same buffer was used in the reservoirs, whereas the heat precipitates were dissolved in a 0.03 M buffer of the same pH containing 8 M urea, creating a conducting gap in the sample chamber The samples were electrophoresed at a constant ltage of 50 V for 45 min, usually with the anode t the lower end.

Molecular W eight Determinations

The following marker proteins were bought from Boehringer Mannheim, West-Germany: Bovine se

rum albumin, mol.wt. 67,000, bovine pancreatic chymotrypsinogen A, mol.wt. 25 000 horse myoglobin, mol.wt. 17,800 and horse heart cytochrome C, mol.wt. 12,400 Electrophoresis was done in 15 per cent polyacrylamide gels and in tubes 12 cm long, but otherwise as described under analytical gel electrophoresis.

RESULTS

Heating of staphylococcal culture fluids to 60 C inactivated the α haemolysin, and a visible precipitate was formed. The loss of haemolytic activity was dependent on the pH of the solution (Fig 1) The inactivation was rapid and complete at a pH of 4–5 while only partial inactivation took place at pH 7 (Fig. 2) Insignificant amounts of protein and no haemolytic material could be extracted from the precipitates with buffers from pH 4 0 to 9.0 while complete solution and high haemolytic activity was achieved by adding 8 M urea. Analysis by SDS polyacrylamide electrophoresis of the precipitates formed at various pH revealed a difference in protein pattern (Fig. 3) At pH 6 (Fig 3 c) and 7 (Fig 3 d) the precipitate contained one major protein which on further purification was shown to be α toxin. Precipitation at more alkaline or acid pH gave a more complex protein pattern. A comparison of the precipitates at pH 5 0 (Fig. 3 b) and 4.0 (Fig. 3 a) in gel diffusion showed that the latter contained a strong precipitinogen which was not related to α toxin. Therefore, adjusting the supernatants to pH 5 0 and heating them to 60 C for 30 min was chosen as *the standard procedure for isolation of the* α toxin.

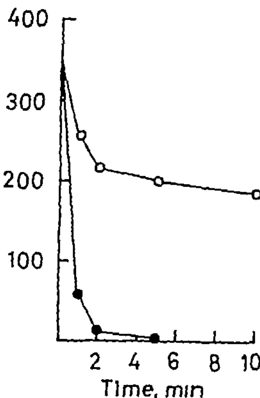


Fig 2 Heat inactivation at 60° C of staphylococcal α-toxin at pH 7 0 (○—○) and pH 4.0 (●—●)

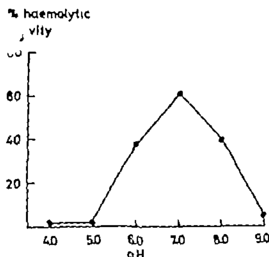


Fig 1 Heat inactivation of staphylococcal α-toxin at varying pH. Samples were heated to 60° C for 5 min. The haemolytic activity after heating was recorded in per cent of the titre before heating.

Purification of α Toxin by Electrophoresis at the Isoelectric Point

In an earlier work (5) the major component of α toxin had a pI of 8 6, while a minor fraction had a pI of 7 4 Heat-precipitates were dissolved in borate buffer 0.05 M pH 8.6, containing 8 M urea. The solutions

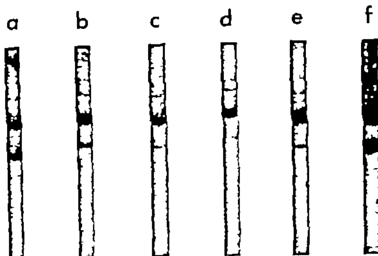


Fig 3 SDS polyacrylamide (15 per cent) electrophoresis of heat precipitates (60 C – 5 min) formed at various pH values. a. pH 4 0, b pH 5 0 c pH 6.0 d pH 7.0 e: pH 8.0 f pH 9.0.

were strongly haemolytic with a specific activity of about 10,000 haemolytic units per mg protein. In order to obtain a rapid separation by electrophoresis at the isoelectric point, the tubes for polyacrylamide electrophoresis were used. The concentration of protein in the sample was usually 1 to 2 mg per ml and the electrophoretic runs were done for 45 min at a constant voltage of 50 V The pH of the sample chamber increased by less than 0 1 pH unit during the run and 50–80 per cent of the haemolytic activity remained in the chamber Analysis of the content by polyacrylamide gel electrophoresis with SDS showed that only one protein remained (Fig 4) The mol.wt. was 39,000 and only one precipitation line was formed on double diffusion in agar against standard serum. The protein showed the characteristic property of α toxin, being strongly haemolytic on rabbit erythrocytes with a specific activity of 25,000 haemolytic units per mg protein.

The electrophoretic separation at the isoelectric point was indifferent to the direction of the current. Application of the anode to the bottom end of the gels was preferred since most of the contaminating proteins moved into the gel and could be eluted after separation. Furthermore the front was marked by a strong, brown pigment and the electrophoretic runs could be monitored by this. With the current in the other direction, only one protein moved into the gels, whereas the rest migrated to the upper reservoir indicating a pI below 8 6 for the majority of the extracellular proteins There was no cathodic migration of α toxin at pH 8 6 under these conditions.

With the anode at the bottom end of the tubes, haemolytic activity could be eluted from a precipitate at the top of the gels and 3 mm below with 8 M urea. The precipitate



Fig 4 α-Toxin purified by electrophoresis at pH 8.6.

contained, in addition to α toxin, several other proteins and notably more low-molecular peptides than in the applied material, indicating high proteolytic activity. The combined haemolytic activity of the two fractions eluted from the gels amounted to less than 10 per cent of the haemolytic activity applied in the separating chamber. The α toxin migrating anodically at pH 8.6 might represent a toxin component with a pI of 7.4. Separation of crude heat precipitates was therefore performed at this pH to examine this possibility and α toxin amounting to 10–20 per cent of the total activity was found, together with other proteins in the separating chamber. Haemolytic material eluted from gels after separations at pH 8.6 also contained electrophoretically immobile toxin at pH 7.4.

Attempts to Dissociate α Toxin

The molecular weight of the toxin isolated by this method was slightly higher than values reported by most workers. To exclude the existence of a dimer dissociation was attempted. Purified toxin in the presence of SDS (1 per cent w/v) was boiled for 2 min at neutrality with 0.2 N NaOH, 0.2 N HCl and formic acid (1 M). The toxin resisted these treatments. Furthermore, dissociation with SDS under reducing conditions (1 mM mercaptoethanol and 8 M urea) did not affect the migration rate in SDS polyacrylamide gel electrophoresis, indicating that the toxin existed as a single polypeptide chain.

A Protein Related to α Toxin

The α toxin with mol.wt. 39 000 was usually found in the pure state when separation was performed at pH 8.6. If the crude material had been stored in the dissolved state for more than 24 h at room temperature an additional protein with mol.wt. 27,500 was found. The optimum condition for separation of this protein was at pH 8.4 and varying amounts of α toxin with mol.wt. 39,000 were always present. The mixtures were haemolytic, but with a lower activity than the pure form of α toxin with a molecular weight of 39 000. When preparative electrophoresis was done at pH 7.2–7.4 a fraction of the protein with mol.wt. 27,500 was immobile at this pH, indicating that this protein also existed in at least two charged forms. On gel diffusion in agar mixtures of the two proteins gave two protein lines which merged with the single line from purified α toxin of molecular weight 39,000.

Stability of the Toxin

A darkening of the heat precipitate was observed during storage at –20 °C, but the haemolytic activity remained unchanged after dissociation in urea. Purified toxins remained stable in the frozen state in 8 M urea for at least 3 months. On storage in 8 M urea at 4 °C, a gradual increase in pH of the solution took place with a concomitant inactivation of the toxin.

DISCUSSION

The increasing rate of inactivation of α toxin at temperatures from 0 to 60 °C probably is a consequence of the hydrophobic reactivity of the protein (5). Heat precipitation at an acid pH was a convenient initial purification step since both concentration and considerable purification were achieved by this simple procedure. The heat precipitates could be stored in the frozen state for long periods of time without loss of haemolytic activity. Proteolytic degradation of the precipitate was, however, evident when kept in suspension at room temperature. This is in agreement with the findings of *Six & Harshman* (9) of proteolytic activity in their partially purified α-toxin.

The variation in protein composition of the heat precipitates at different pH might be regarded as due to pH-dependent coprecipitation with α toxin. Several of the proteins did not precipitate when heated in the absence of α toxin. The maximal precipitation at pH 4.0–5.0 would correspond to the formation of a complex precipitate contain-

ing a toxin in this pH region on purification with isoelectric focusing as reported by several groups (5 8, 10) The precipitate was completely dissolved by treatment with 8 M urea, with the release of haemolytic activity This material evidently differs from that obtained by the commonly used extraction of ammonium sulphate precipitates with non-dissociating buffers, where only a partial solution is obtained. In addition to the advantage of simplicity the present method, due to its rapidity reduced inactivation of the toxin. It was important to create a conductivity gap by keeping the ionic strength in the sample chamber lower than in the reservoir and the gels. An equal ionic strength throughout the system retarded the separation and caused loss of toxin, probably due to diffusion.

Like the isoelectric focusing method, a minor component of α toxin with a pI of 7 4 could be demonstrated by the present method. The minor component accounted for 10–15 per cent of the total haemolytic activity The presence of this component has been attributed to the action of an extracellular staphylococcal arginase attacking arginine within the toxin molecule (6) The toxin with a pI of 7 4 was not obtained in its pure form by the present method alone, the contaminating proteins having a pI very close to that of the minor toxin component.

The protein with mol.wt. 27,500 showed an antigenic relationship to the toxin with mol.wt. 39,000. Furthermore, it appeared in at least two charged forms. The protein therefore resembled the α-toxin with a molecular weight of 27,500 and the pI of the major component of 8.4 and of the minor component of 7.2 reported by *Six & Harshman* (9) It differed, however in one important respect, in that it had low or no haemolytic activity The protein has been shown earlier to appear in a non-haemolytic fraction with a pI of 8.4 on isoelectric focusing (5)

The relationship between the two proteins could be explained in two ways. The protein with mol.wt. 27,500 might be a degradation product of the toxin of molecular weight 39 000 Two charged forms of the degradation product would then be expected. A second possibility would be that the toxin with mol.wt. 39,000 was a dimer of a degraded protein with mol wt. 27,500. The increase in the S value of the toxin from 1 7 to 3 0 on storage reported by *Wiseman et al* (12) would support this idea. The toxin with mol wt. 39 000 was not, however cleaved by any of the conventional methods for dissociation tested.

Work in progress supports the view that the pure protein with mol.wt. 27,500 is non-haemolytic and appears by degradation of the toxin with a molecular weight of 39 000 by a chymotrypsin-like enzyme.

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SPONTANEOUS α-TOXIN MUTANTS OF *STAPHYLOCOCCUS AUREUS*

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Dalen, A. B. Spontaneous α-toxin mutants of *Staphylococcus aureus*. Acta path. microbiol. scand. Sect. B, 84 333–338, 1976.

Prolonged cultivation of strain Wood 46 in fluid cultures resulted in a selection of mutants with low or no haemolytic activity In one group of mutants, four out of five strains showed no production of α-toxin when examined by polyacrylamide gel electrophoresis and by double diffusion in agar Two major extracellular proteins which have been identified by other methods as degradation products of α-toxin were also absent. The absence of α-toxin did not affect growth in fluid or solid media. Fibrinolysin was produced by these mutants but at a much lower rate than by the wild type. A second group of mutants was characterized by a slow rate of growth on rabbit blood agar and showed a heterogeneous extracellular protein pattern. These mutants had a high growth rate in fluid medium consisting of acid hydrolyzed proteins. Production of fibrinolysin was absent or low in three out of four mutants in the second group. The slow growth and low production of α-haemolysin in rabbit blood agar probably was caused by deficient extracellular proteolytic activity of the mutants.

Key words Staphylococcal α-toxin spontaneous mutants.

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Staphylococcal α toxin is an extracellular protein produced by most human pathogenic strains. *McClatchy & Rosenblum* (7 8) studied non-haemolytic mutants obtained by induction with ultraviolet light and nitrous acid. The results of two-point reciprocal transductions placed the mutants into two genetic groups. One group was postulated to be α-toxin structural mutants. In the second group, a mutation of a pleiotropic gene si multaneously affecting the synthesis of both α toxin and fibrinolysin apparently occurred. They also showed that mutations which altered the α toxin molecule affected its lethal, dermonecrotic and haemolytic activities either selectively or to varying degree.

Strain Wood 46 is widely used as a source of α toxin. Non-haemolytic variants readily appear when the strain is grown in fluid cultures for long periods (5 6) The production of α toxin and other extracellular proteins in such mutants is reported in this work.

MATERIALS AND METHODS

Organisms

Staphylococcus aureus strain Wood 46 phage-type 42 D (1000 RTD) which has been cultured for several years in this laboratory was used It was propagated on human blood agar plated out on rabbit blood agar every two months, and the colonies which showed wide zones of haemolysis were used for further propagation

Selection of Mutants

Stagnant cultures of bacteria were made in tubes containing 1 ml of a medium consisting of (per litre): casamino acids (vitamine-free) (Difco Laboratories, Detroit Mich., USA) 20 g thiamine hydrochloride, 0 1 mg nicotinic acid 6.0 mg glycerol 0.5 per cent (v/v) H_3PO_4, 1 0 g. The pH was adjusted to 7 1 and the medium was sterilized by Millipore filtration, pore size 0.45 µ. Passages of bacteria to fresh medium were done every second day until the fifth passage. The bacteria were then streaked out on rabbit blood agar plates and incubated at 37 C for 24 h. Colonies showing low or no haemolytic activity were selected for further examination.

Phage Typing

The typing phages used were those recommended by the Subcommittee on the Phage Typing of Staphylococci (6th Meeting September 1974)

Haemolytic Assay

Rabbit erythrocytes, collected fresh daily in Alsever's solution, were washed twice in phosphate buffered saline (PBS) containing sodium chloride, 0.082 M and Sorensen phosphate buffer 0.03 M (pH 7.0) then suspended in PBS to a concentration of 2 per cent. Twofold serial doubling dilutions (0.5 ml) of toxin were made in PBS, and 0.5 ml of erythrocyte suspension was added to each tube The tubes were incubated at 37 C for 30 min, and 50 per cent haemolysis was read visually

P eparation of Extracell lar Proteins

The Casamino acid medium was supplemented th an evaporate of 50 per cent (v/) ethanolic act f 10 g yeast autolysate (Oxoid) per litre medium. Cultures were incubated on a rotary shaker (Controlled Environment, Incubator Shaker New Brunswick Scientific, New Brunswick, N J USA) 150 strokes per min at 37 C. A starter culture was grown overnight, and 10 ml of bacterial suspension was used to inoculate larger cultures distributed in 300 ml aliquots in 2 litre flasks. After overnight incubation bacteria were removed by centrifugation and by filtration on Millipore filters, pore size 0 45 µ. Proteins were precipitated by adding trichloroacetic acid (TCA) to a concentration of 5 per cent (w/v) The precipitates were collected by centrifugation and then washed twice in a solution containing ethanol (50 per cent) and borate buffer 0 03 M, pH 8.3 (50 per cent) Precipitates to be examined for haemolytic activity or precipitation in gel, were dissolved in borate buffer (0.03 M pH 8.3) containing 8 M urea. The precipitates to be examined by polyacrylamide gel electrophoresis, were dissolved in 2 per cent sodium dodecyl sulphate (SDS)

The presence of heat-precipitable proteins in the extracellular fluids was examined by heating samples at 60 C in a water bath for 30 min at pH 4.0 or 7.0. The precipitates formed were treated in the same manner as the TCA precipitates.

Double Diffusion Tests

Gel diffusion was carried out in Petri dishes containing a 3 mm thick layer of 1 per cent agarose (Behringwerke) in phosphate buffer (0 05 M, pH 7 0) with a 3 mm centre serum well and 6 peripheral antigen wells of the same size placed 7 mm from the centre well. The antiserum used was an international standard for staphylococcal anti-toxin, 1 ml containing 20 I.U obtained from Statens Seruminstitut, Copenhagen (batch no. 88)

A alytical Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as described previously (4) The gels contained 15 per cent (w/v) acrylamide and the same buffer (0 37 M triglycine, pH 9.5) containing 0 1 per cent SDS (w/) was used in the gel and in the electrode vessels.

Tests for Coagulase Fibrinolysin Lipase and Hyaluronate Lyase

Coagulase Routine tube tests were performed using rabbit plasma diluted 1 in 4 in broth

Fibrinolysin Twelve per cent citrated human plasma was added to nutrient agar and placed in a water bath at 56 C for 15 min. The mixture was then poured into Petri dishes. The procedure gave an opaque medium, and fibrinolytic colonies produced clearing after overnight incubation at 37 C. Fibrinolysin was extracted from TCA precipitates by means of trischloride buffer (0.05 M pH 7.5) The fibrinolytic activity of these extracts was measured by applying 20 µl into the wells of the agar and then measuring the clearing zones after 24 h incubation at 37 C

Lipase The staphylococcal strains were spot inoculated on nutrient agar containing Tween 80 and $CaCl_2$ as described by *Sierr* (10)

Hyaluronate lyase The decapsulation test recommended by *Oehring* (9) was used. A strain of *Bacterium anitratum* was inoculated on blood agar plates followed by spot-inoculation of the staphylococcal strains. The results were read after incubation at 37 C for 18–22 h.

RESULTS

The bacterial population was dominated by variants which showed weak or no haemolytic activity on rabbit agar plates 24 h after five passages in fluid cultures. After prolonged

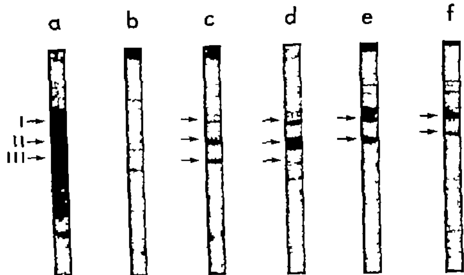


Fig 1 Polyacrylamide gel electrophoresis with SDS of TCA precipitates of extracellular staphylococcal proteins. a: wild strain, Wood 46. b one of four identical group I mutants with no α-toxin production. c: group I mutant with low α-toxin production. d: group II mutant 1 e: group II mutant 2 and 3 f: group II mutant 4 I α-toxin mol.wt. 39,000. II intermediate from α-toxin mol.wt. 27,500. III intermediate from α-toxin mol wt. 23,500

incubations (48 to 72 h) weak zones of haemolysis were observed both on human and rabbit blood agar plates. From the growth on human and rabbit agar blood plates, the mutants could be classified onto two groups. Mutants from group I produced colonies equal in size and in appearance to the wild strains on both types of media. Mutants from group II resembled the wild type in its growth on human blood agar plates, whereas the colonies were much smaller and of a greyish colour on the rabbit blood agar plates. Five independent isolates of group I mutants and four isolates of group II mutants were examined further

Phage Type and Extracellular Enzyme Activities

The nine mutants were all of the same phage type as the wild strain (42 D/10000) They all produced coagulase, lipase and hyaluronate lyase. Fibrinolytic activity was detectable around the colonies of all the mutants of group I. The fibrinolytic zones were less than 1 mm around the colonies in two of the four mutants of group II and undetectable in one of the mutants. The test for fibrinolytic activity of the TCA precipitates was positive in two of the mutants of group I where an activity of 5–10 per cent of that of the wild type was observed. No fibrinolytic activity was detected in the precipitates from the other seven mutants.

The haemolytic activities on rabbit erythrocytes of the supernatants from fluid cultures were examined after overnight incubation. One of the mutants of group I (Gr I, 1) showed a haemolytic activity of 12 per cent compared with that of the wild type, while in the four other mutants of this group only 3 per cent of the activity of the wild type was found. The mutants of group II produced more haemolytic material, and the activity varied from 25 to 80 per cent of that of the wild type.

Extracellular Proteins in TCA Precipitates

TCA precipitates of supernatants from cultures of Wood 46 contained a number of proteins (Fig 1 a) α Toxin in its haemolytic form has a molecular weight of 39,000 (arrow I) A non-haemolytic protein with

mol.wt 27,500 has been shown to appear after proteolysis of α toxin by an extracellular chymotrypsin-like enzyme (arrow II) (5) The relative amounts of the two proteins vary inversely and the amount of the protein with mol.wt. 27,500 was high in the preparation shown in the figure. A protein with mol.wt. 23,500 (arrow III) appears after degradation of the protein with mol.wt. 27,500 by a trypsin-like enzyme (5) The protein composition of the TCA precipitates was identical in four of the five strains from group I (Fig 1 b) α Toxin and the two peptides were absent. The protein pattern of the fifth mutant of group I (Fig 1 c) was more like the wild type, both α toxin and its two degradative products being present.

Polyacrylamide electrophoresis of the precipitates from the group II mutants revealed a heterogeneous extracellular protein pattern. One of the mutants resembled the wild type, in that α toxin and its two degradative intermediates were present (Fig. 1 d) Two of the mutants were identical to each other and differed from the wild strain mainly in the absence of the protein with mol.wt. 23,500 (Fig 1 e) The fourth mutant lacked the protein with mol.wt. 23,500 and appeared to be deficient in the production of a protein complex with mol.wt. 40 000 to 45 000 (Fig 1 f)

Heat-Precipitable Extracellular Proteins

Heat precipitates at pH 7 0 of supernatants from Wood 46 contained four proteins of which the major protein was the α toxin (Fig 2 a) Precipitates from the group I mutants contained three proteins with molecular weights corresponding to three proteins from the wild strain (Fig 2 b) One of the proteins had the same molecular weight as α toxin. The relative amounts of this protein was lower than that of α toxin in the wild strain. Attempts to identify this protein as α-toxin were not successful. Heat precipitates from the four identical group I mutants were dissolved in 8 M urea. The haemolytic activity was low and no reaction of identity was obtained with purified α toxin on gel diffusion in agar

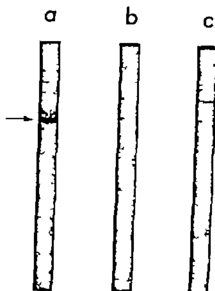


Fig 2 Polyacrylamide gel electrophoresis with SDS of heat precipitates of extracellular staphylococcal proteins. a: wild strain, Wood 46. α-Toxin *mol.wt. 39 000 is indicated by an arrow b: one of* four identical mutants of group I. c group II mutants.

Heating of fluids, from cultures of group II mutants produced small amounts of precipitates. The protein composition of the precipitates was, however very similar to that of the wild type (Fig 2 c)

Gel Diffusion in Agar

Three strong precipitation lines were produced using TCA precipitates from Wood 46 as the antigen (Fig 3) Pure α toxin gave a reaction of identity with the line in the middle. This precipitation line was completely

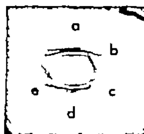


Fig 3 Gel diffusion in agar of TCA precipitates of extracellular staphylococcal proteins. and d: wild strain, Wood 46 b, c and e three group I spontaneous mutants not producing α-toxin.

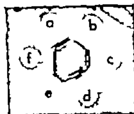


Fig. 4. Gel diffusion in agar of TCA precipitates of extracellular staphylococcal proteins. a and d: wild strain, Wood 46. b, c and f: three different group II spontaneous mutants with low production of α-toxin. e: group I spontaneous mutant with no production of α-toxin.

lacking using similar antigens from four of the five mutants of group I (Fig. 3). The group I mutant which possessed some α toxin production and all the mutants of group II produced a precipitation line which gave a reaction of identity with the wild type α-toxin (Fig. 4).

Immunodiffusion also indicated differences in the composition of the extracellular proteins of the mutants of group II. The strong outer precipitation line of the wild type was absent in one of the mutants (Fig. 4 b), but was present in another (Fig. 4 f).

DISCUSSION

Spontaneous mutations represent a heterogeneous class and differ from those induced by ultraviolet light and mutagens such as nitrous acid in many systems. Of the 20 non-haemolytic mutants of *Staphylococcal aureus* obtained by induction with ultraviolet light and nitrous acid, 13 strains were completely deficient in the synthesis of α-toxin or immunologically related proteins (7). These mutants resembled four of the mutants of our group I where no α-toxin was produced. A structural gene mutation would seem likely. None of these were completely lacking in the production of haemolytic material. Haemolysis was observed both on human and rabbit blood agar plates and was probably caused by the delta haemolysin demonstrated in strain Wood 46 (2). The fifth mutant of group I resembled the wild strain, but had a lower production of α toxin. There was no evidence of a difference in the molecular weight of the mutant toxin from that of the wild type toxin, and the same degradation pattern was observed.

The lack of α-toxin did not affect the growth rate in fluid cultures or on solid media. The functional role of α-toxin in staphylococci has not been established, but this finding would indicate that α-toxin is not essential for the growth of this strain.

The extracellular proteolytic degradation of α toxin resulting in an accumulation of relatively stable intermediates is of great importance in the evaluation of the extracellular protein pattern of staphylococci revealed by polyacrylamide gel electrophoresis (5). The absence of the two intermediates with mol.wt. 27,500 and 23,500 in the four identical group I mutants appears to confirm the pathway of degradation of α toxin discussed in an earlier study (5). Strains of pathogenic staphylococci elaborate a higher number of extracellular protein species than non-pathogenic strains (1). In this context, the loss of several extracellular proteins with varying molecular weights due to the mutation of a single structural gene should be kept in mind.

The second type of mutants appeared to represent a heterogeneous group. These mutants resembled a UV mutant studied by *Bernheimer et al.* (2). Their mutant was not haemolytic on rabbit blood agar, but α-toxin was produced in fluid cultures. The toxin seemed to be similar to that produced by the wild strain, but was elaborated in smaller quantities. The discrepancy in the production of α toxin on solid and fluid media might be related to the poor growth of the mutants on rabbit blood agar plates. The superior growth on human blood agar is not understood. The small amounts of low molecular peptides found in the TCA precipitates and the absence of α toxin related protein with mol.wt. 23,500 might be a clue to solving the problem. The protein with mol.wt. 23,500 has been found to be produced due to the action of a trypsin-like enzyme on the protein with mol.wt. 27,500 (5). A deficiency of the extra-

cellular proteolytic activity might therefore be characteristic of the group II mutants. This would explain that the growth rate and the α toxin production were comparable to that of the wild strain in the Casamino acid medium, which consisted of acid hydrolysed proteins. Poor growth on solid media containing proteins would be expected due to insufficient availability of amino acids.

The genetic relationship between fibrinolysin and α toxin observed in induced mutants (8) was less clear in the spontaneous mutants. In three of the four mutants of group II, the production of fibrinolysin was negligible or absent both in fluid cultures and on growth on solid media. All the five mutants of group I produced fibrinolytic material when grown on solid media. However judging from the amounts found in the TCA precipitates, the quantities were far below that produced by the wild strain. Therefore, even in apparently structural mutants of α-toxin, the amount of fibrinolysin produced is affected.

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THE HAEMOLYTIC ACTIVITY OF *HAEMOPHILUS* SPECIES

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Kilian, M. The haemolytic activity of *Haemophilus* species. Acta path. microbiol. scand. Sect. B, 84 339–341 1976

The importance of the species of blood employed for detection of haemolysis in seventy-seven Haemophilus strains of human and porcine origin was studied. Significant differences in the visibility of haemolytic zones obtained on the different blood agar media were observed. In decreasing order the suitability of the species of blood was: calf sheep, human, rabbit, poultry and horse blood. On plates containing washed sheep or calf red cells the haemolysin of all 36 strains of *Haemophilus pleuropneumoniae* acted synergistically with the β-toxin of the *Staphylococcus aureus* strain used as "feeder strain" giving rise to lytic phenomenon resembling the CAMP reaction.

Key words: Haemophilus haemolysis; taxonomy

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The haemolytic activity of certain haemophili constitutes one of the primary distinguishing characteristics used to define the species *Haemophilus haemolyticus* *H parahaemolyticus* (5) and *H paraphrohaemolyticus* (8) In addition, *H pleuropneumoniae* (*H parahaemolyticus var suis*) is known to attack red blood cells. On blood agar media, a zone of β-haemolysis surrounds colonies of freshly-isolated strains of these species. However after a few subcultures the haemolytic activity is often reduced or lost, creating identification difficulties (3)

This study is an attempt to evaluate the importance of the species of blood employed for detection of haemolysis, and describes a distinctive haemolytic activity of *H pleuropneumoniae*

MATERIALS AND METHODS

Preparation of blood agar media. The media used were 5 per cent (v/v) human, horse, calf, rabbit, sheep and poultry blood agar (Blood Agar Base, Difco) prepared with red cells from freshly drawn blood. The erythrocytes were washed once and resuspended in sterile saline (0.85 per cent w/v) to the original volume of the blood

Bacterial strains. Seventy-seven haemolytic and non-haemolytic Haemophilus strains were included in the study Nomenclature and identification of the strains are in accordance with a previous proposal (5) The 36 porcine strains of *H pleuropneumoniae* included 1 strain of serotype 1 20 strains of serotype 2, 8 strains of serotype 3 4 strains of serotype K 17 one strain of serotype K 98 and 2 non-typable strains. These strains were isolated in Denmark, Switzerland, Canada Holland, Sweden, Finland, the Republic of China, Taiwan and the U.S.A. Fourteen of these strains (HK 351 361 HK 405-407) have been described previously (3) The strains isolated from man were: *H haemolyticus* strains HK 5 12, 29 75 and 386 (NCTC 10659) *H parainfluenzae* bio-

type I strains HK 18, 47 79 88, 90 96 and 109 *H parainfluenzae* biotype II strains HK 23 31 60 64 6 81 95 117 120 122, 335 336 410 (NCTC 10665) *H parainfluenzae* biotype III strains HK 19 22 48 51 65 121 137 261 303 304 305 330 334 385 (NCTC 8479) 404 (NCTC 10794) and 411 (NCTC 10670) Twenty of the strains belonging to *H parainfluenzae* biotype II and III caused β-haemolysis on horse blood agar (3) The characteristics and origins of these human strains have been described previously (3)

As "feeder strains" two different staphylococcus strains were used: *S aureus* strain 5627/65 (β-toxinogenic, phage type 73A u) and a non-haemolytic staphylococcus strain MK 78.

Comparison of species of blood. For comparison of haemolytic activity against different species of blood, each strain was cultivated on the respective blood agar media. The plates were inoculated with a loopful of Levinthal broth cultures of the respective Haemophilus strains and then the plates were immediately streaked with staphylococcus strain MK 78.

Detection of the CAMP-reaction A streak was made across the centre of each blood agar plate with a loopful of a culture of *S aureus* strain 5627/65. At right angles to this were streaked the strains of haemophili to be tested, care being taken to avoid contact with the staphylococcus. Six strains were tested on each plate (Fig. 1) This test was performed on all types of blood agar

After incubation for 2 days in air plus 10 per cent CO_2 at 37 C, the degree of haemolysis was recorded

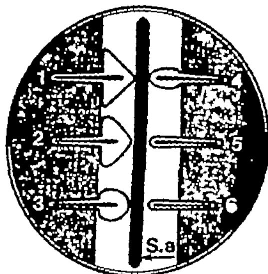


Fig 1 Lytic phenomena caused by six haemolytic Haemophilus strains streaked and grown at right angles to a streak of a β-toxinogenic *Staphylococcus aureus* (S.a.) strain on sheep blood agar 1 *H pleuropneumoniae* serotype 2 2 *H pleuropneumoniae* serotype 1 3 *H pleuropneumoniae* serotype 3 4 *H pleuropneumoniae* serotype K 17 5 *H haemolyticus* 6 *H parainfluenzae* (haemolytic strain)

RESULTS

Significant differences in the sizes of haemolytic zones obtained on the different blood agar media were observed. The largest and clearest zones were obtained on calf blood agar The suitability of the different species of blood for the detection of haemolysis was, in decreasing order calf sheep human, rabbit, poultry and horse blood. Several strains showing only faint haemolysis on horse blood agar produced large clear zones on calf blood agar Human strains previously found to be non-haemolytic on horse blood agar remained non-haemolytic on all media. These strains included six strains which had lost the haemolytic activity on subculture (3) However five strains of *H pleuropneumoniae* which showed no detectable haemolysis on horse blood agar produced clearly visible lytic zones on calf and sheep blood agar

Cultures of all 36 strains of *H pleuropneumoniae* produced increased zones of haemolysis on sheep and calf blood agar within the zones of partial lysis surrounding the β-toxinogenic feeder strain. This enhanced haemolytic effect was not observed on agar media containing red cells of any of the other species of blood employed. None of the strains of human origin caused a similar lytic phenomenon. Fig 1 illustrates the different haemolytic patterns obtained on sheep agar by growing various Haemophilus strains at right angles to a streak culture of the β toxin producing staphylococcus. The figure shows the characteristic triangular lytic zone observed in all strains of *H pleuropneumoniae* serotype 2 and the strain of serotype 1 All other strains of *H pleuropneumoniae* produced a rounded extension of the lytic zone (Fig. 1) within the zone surrounding the feeder strain.

DISCUSSION

The results of this study emphasize the importance of the species of red cells used for the detection of haemolytic activity of Haemophilus strains. It is noteworthy that the weakest reactions were obtained on horse blood agar which is used commonly for this purpose. Thus five strains of *H. pleuropneumoniae* previously reported to have lost their haemolytic activity on horse blood agar (3) produced visible lytic zones on calf and sheep blood agar.

In contrast to the human haemolytic strains all strains of *H. pleuropneumoniae* possessed the ability to potentiate the partial lysis of sheep and calf red cells caused by a β-toxinogenic staphylococcus employed as feeder strain. This lytic phenomenon resembles the CAMP-reaction (4) as originally observed in strain of *Streptococcus agalactiae* (1). In accordance with the CAMP reaction, the lytic phenomenon produced by strains of *H. pleuropneumoniae* was only seen on sheep or calf blood agar. More recently a similar lytic phenomenon has been observed in a number of animal pathogens including some group C and G streptococci from bovine and canine sources (6), some corynebacteria (2, 7) and *Pasteurella haemolytica* (2). The fact that the CAMP reaction was only encountered in strains of *H. pleuropneumoniae* suggests that this property may be of value as a distinguishing characteristic. It is noteworthy that the pattern of the lytic phenomenon in strains of *H. pleuropneumoniae* varied among the different serotypes. The findings obtained in this study support further a previous suggestion (3) that haemolytic V-dependent Haemophilus strains of porcine origin should not be classified with haemolytic strains of human origin.

The strains of *H. pleuropneumoniae* were kindly provided by Dr. *R. Nielsen*, Statens Veterinære Serumlaboratorium, Copenhagen, and Professor Dr. *J. Nicolet*, Veterinär Bakteriologisches Institut, Bern. The β-toxinogenic staphylococcus strain was received from Dr. *I. Lind*, Statens Seruminstitut, Copenhagen.

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AGE- AND SEX-RELATED DISTRIBUTION OF ANTIBODIES TO HEPATITIS B SURFACE AND CORE ANTIGENS IN A *SWEDISH POPULATION*

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Hansson, B. G Age and sex-related distribution of antibodies to hepatitis B surface and core antigens in a Swedish population. Acta path. microbiol. scand. Sect. B, 84 342–346 1976

Serum samples from people born between the years 1895 and 1970 were collected and tested for antibody to hepatitis B surface antigen (anti-HB) by passive haemagglutination (PHA) and radioimmunoassay (RIA) and for antibody to hepatitis B core antigen (anti HB) by immunoelectroosmophoresis (IEOP) Neither anti-HB nor anti-HB was detected in the serum from anyone under 15 years of age. The prevalence of anti-HB$_s$ and anti-HB showed peaks of 9.2 and 8.3 per cent, respectively in the age group of 40–49 years The distribution of antibody was equal between men and women. Eighty per cent of the sera with anti-HB were also positive for anti-HB All sera positive for anti-HB also contained anti-HB

Key words Anti-hepatitis B core anti-hepatitis B surface normal population.

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Two separate viral antigens, hepatitis B surface antigen (HB$_s$Ag) and hepatitis B core antigen (HB$_c$Ag) have been found associated with the hepatitis B virus (1 12) The outer component of the Dane particles, the 20 nm spheres and the filamentous forms all contain the HB$_s$Ag The internal core of the Dane particle is characterized by the HB$_c$Ag This antigen is also present in the nuclei of infected hepatocytes.

The presence of antibody to hepatitis B surface antigen (anti-HB) is an indication of prior contact with hepatitis B virus. Since the development of sensitive methods such as passive haemagglutination (PHA) (27) and radioimmunoassay (RIA) (4 8, 15 28) it has become possible to demonstrate anti-HB$_s$ in a high percentage of patients who have recovered from acute hepatitis B infections (2 16 22) In West European and American populations without a known history of clinical hepatitis, prevalences of anti-HB$_s$ of between 5 and 20 per cent have been reported (15 17 19 22, 23 24 26) while in highly endemic areas anti-HB$_s$ has been detected in more than half of the population (3 5 6)

Antibody to hepatitis B core antigen (anti HB$_c$) has been demonstrated in virtually all HB$_s$Ag-carriers and patients with acute hepatitis B. After recovery from hepatitis B infection the titres of anti HB$_c$ have been reported to fall and anti-HB$_c$ has been considered shorter lived than anti HB$_s$ (12, 15) The

prevalence of anti-HB_s in different age groups of a normal population has so far not been investigated.

In this study the occurrence of anti HB_s and anti-HB_s related to age and sex in a Swedish population is presented.

MATERIALS AND METHODS

Serum samples. During 1973 and 1974 serum samples were obtained from outpatients of the Orthopedic Clinic, Malmö General Hospital. These patients were considered representative of the general population regarding history of hepatitis infections and had no indications of disease other than the orthopedic one for which they were consulting. Sera were collected from six female and six male patients from each birth year between 1895 and 1970 with a few exceptions in the younger and older age groups. A total of 889 sera were collected and stored at –20 C until tested.

Passive haemagglutination (PHA). Anti-HB was tested for by PHA according to the method described by *Vyas & Shulman* (27). A titre of 1/8 or greater was considered as a positive reaction. The red cells were coated with purified HB_sAg which had been prepared from a pool of sera containing HB_sAg/ad and HB Ag/ay by treatment with fluorocarbon followed by two consecutive isopycnic bandings in caesium chloride gradients and one rate zonal centrifugation in a sucrose gradient. Three different batches of purified antigen were prepared. All sera in this investigation were tested with erythrocytes coated with two of these antigen preparations.

Radioimmunoassay (RIA). A newly developed solid-phase RIA test (Ausab*) was used for anti-HB determination as described by *Lange et al.* (18).

Specificity tests. The testing of a limited number of blood donor sera with erythrocytes previously coated with one batch of purified HB_sAg showed one per cent false positive reactions while red cells coated with two other batches of HB_sAg gave about 15 per cent false positives. This emphasizes the importance of performing specificity analysis on all sera positive by PHA. In these tests the sera were mixed and incubated for two hours at room temperature and over night at +4 C with a serum pool containing HB_sAg/ad and HB_sAg/ay or with a serum negative for HB_sAg and anti-HB by RIA. The samples were considered confirmed positive for anti-HB if they became negative by PHA after incubation with HB_sAg and remained positive after incubation with negative serum. All sera positive

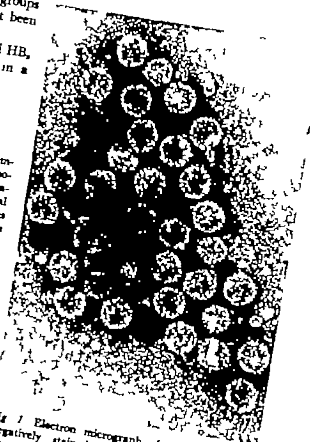


Fig. 1. Electron micrograph of purified HB_sAg. Negatively stained with phosphotungstic acid. Magn. × 280,000.

by RIA were confirmed to contain anti-HB by specificity test.

The testing of two-fold dilutions of three different sera containing anti-HB_s could not demonstrate any difference in sensitivity between PHA and RIA.

Purification of hepatitis B surface antigen (HB_sAg). HB_sAg was purified according to the method of *Hoofnagle et al.* (12) from the liver of a patient who was a chronic carrier of HB_sAg/ay and who in his life time had received two renal transplants. He was treated with immunosuppressive drugs for seven months prior to his death. The liver* was removed at necropsy, frozen and thawed and homogenized in a Waring blender. A suspension of 20 per cent homogenate was made in hypotonic saline and was clarified by centrifugation at 300 g for 30 minutes. The supernatant was centrifuged at 73000 g for 2 hours. The resulting pellet was resuspended, and the HB_sAg was further purified

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* Abbott Laboratories, North Chicago Ill USA

* kindly supplied by Dr *Bengt Lindqvist*, Department of Medicine, University of Umeå.

by isopycnic banding in a caesium chloride gradient. HB_sAg was detected by IEOP using anti HB from NIH, Bethesda, Md. Fractions containing HB_sAg were pooled and dialyzed. Hexagonal shaped, 27 nm particles with distinctive subunits were seen upon electron microscopical examination (Fig. 1) The antigen pool was negative for HB_sAg by RIA (Ausria II) The purity of the HB Ag preparation was also tested through immunizing a rabbit with the antigen. High titre of anti-HB was detected by IEOP but no anti-HB antibodies could be demonstrated by PHA in the serum of the animal. The identity of this preparation as HB_sAg was kindly confirmed by Dr *Robert Gerety* Bureau of Biologics, Bethesda, Md.

Immunoelectroosmophoresis (IEOP) The IEOP technique previously described for HB_sAg and anti HB detection (9) was pplied for the detection of anti-HB using purified HB_sAg.

RESULTS

Erythrocytes coated with two different batches of HB_sAg gave the same results in PHA after confirmatory neutralization tests. Anti HB_s was detected in 40 out of the 889 sera collected at the Orthopedic Clinic. The prevalence of anti HB_s in the different age groups is shown in Fig 2 The youngest male with anti HB_s was 18 and the youngest female with anti HB_s was 15 years of age. The prevalence of anti-HB_s increased with age reaching a maximum of 9.2 per cent (95 per cent confidence limits 4 0–14 4 per cent) in persons 40–49 years of age. Beyond this age the frequency of anti-HB_s decreased and was about four per cent in the age group 70–79 years. The distribution of antibody was equal between men and women. Three hundred ninety-nine randomly selected samples out of the 849 PHA negative sera and the 40 PHA positive samples were tested for anti-HB_s by RIA. Five PHA negative sera were found positive while four PHA-positive sera were found negative by RIA.

Anti HB_s was detected in 32 (80 per cent) of the 40 sera with anti-HB Thirty of these sera were positive for anti HB_s both by PHA and RIA, while the remaining two were only positive by PHA. The 849 sera negative for anti-HB_s were also negative for anti HB_c. The distribution of anti HB_s in the different

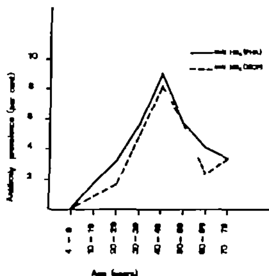


Fig 2 Age distribution of anti-HB and anti-HB in 989 "normal" individuals.

age groups correlated well with that of anti HB_s (Fig. 2)

DISCUSSION

Five out of 399 sera negative for anti-HB_s by PHA were detected positive by RIA. Extrapolating this result to all the 849 PHA negative sera indicates that RIA would be expected to detect anti-HB in 11 of these sera. This would mean an over all increase of the incidence of anti-HB_s from 4.5 to 5 7 per cent. RIA, however failed to detect four out of the totally 40 sera positive by PHA. Consequently if RIA had been used as the only test for anti HB_s it should be estimated to have detected a total of 47 (5.3 per cent) positive sera.

Although the incidence of clinical hepatitis as well as the prevalence of HB_sAg carriers in Sweden are relatively low (10 14 21) 5 4 per cent of the population over 20 years of age in this study had anti HB_s. The majority of these individuals with anti HB_s most likely had subclinical hepatitis B infections.

Hoofnagle et al. (12, 13) detected, by PHA and RIA, anti-HB_s in three to four per cent of normal blood donors. However anti HB_s was found in only one per cent by com-

plement fixation (CF). It is presumed that the lower frequency of anti-HB_c compared to anti-HB_c reported by *Hoofnagle et al.* (12, 13) reflects a declination of the complement fixing in contrast to the precipitating antibodies to HB_cAg or, alternatively, a difference in sensitivity between CF and the IEOP used in this study for the detection of anti-HB_c. It should be pointed out that staining of the electrophoresis plates increased the detection rate of anti-HB_c by about 100 per cent in sera tested here. The importance of staining the electrophoresis plates has previously been emphasized when demonstrating HB_sAg (9) or rota virus (25) by the same technique.

The occurrence of anti-HB_c has been interpreted as a sensitive marker of ongoing or recent virus replication (12, 20). In acute hepatitis B, anti-HB_c has been reported to appear at about the time of onset of clinical symptoms with a gradual decrease in titre after the convalescent period (17). However, in all of 21 patients with acute hepatitis B, who had turned HB_sAg-negative, the present author could detect anti-HB_c five years later (11). In the material studied here the prevalences of anti-HB_s and anti-HB_c have been shown to correlate well in all age groups. Accordingly, it seems justified to presume that anti-HB_c, when detected together with anti-HB_s, is an expression of a clinical or subclinical hepatitis B infection in the past and that anti-HB_c as well as anti-HB persist.

The result of this study showing that no person under 15 years of age had detectable anti-HB or anti-HB_c indicates that hepatitis B in Sweden has been mainly an infection of adults. This observation could possibly support the hypothesis made by *Fulford et al.* (7) that hepatitis B is commonly a sexually transmitted disease. However, this [illegible] be the main route of infection in highly endemic areas, where the incidence of a[illegible] HB [illegible] ready in early childhood [illegible] 3 6 [illegible]. On the other hand, there is no [illegible] explanation for the peak prevalences of [illegible] HB [illegible] anti-HB_c found in the age group of 30–4[illegible] years. The age distribution might indicate changes of the endemicity of hepatitis B virus during the last 75 years. Observations of anti-HB_s maxima at 40 and 60 years of age respectively have previously been reported in other Scandinavian populations (23–26).

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FUNCTIONAL AND ULTRASTRUCTURAL STUDIES OF THE EFFECTS OF HUMAN INTERFERON ON CELL MEMBRANES OF *IN VITRO* CULTURED CELLS

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Degré, M. & Hovig, T. Functional and ultrastructural studies of the effects of human interferon on cell membranes of *in vitro* cultured cells. Acta path. microbiol. scand. Sect. B, 84 347–358 1976.

The effect of human leukocyte interferon on cultured U-amnion cells was examined, and several biological parameters were registered. Multiplication of Vesicular stomatitis virus and the virus-produced cytopathogenic effect was prevented. The growth rate of uninfected cells was reduced, as well as the spontaneous release of ^{3}H-uridine. These effects were observed following treatment with 10 units of interferon per ml. No morphological alterations could be detected by scanning electron microscopy after 24 or 72 hours treatment with up to 2000 units interferon per ml.

Key words Human interferon cell membranes; function ultrastructure.

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Besides its classical antiviral activity interferon seems to have a great variety of biological effects in the whole organism and on cultured cells inhibition of growth of normal as well as of tumour cells (2, 8, 11 17 18) enhanced phagocytosis by macrophages (13) enhanced production of interferon by priming (20) enhanced cellular sensitivity to the toxic effects of vaccinia virus (12) and double stranded polynucleotides (21) enhanced cytotoxicity of lymphocytes (16) and others. It is generally accepted, although not finally proved, that the mechanism of antiviral activity is a preferential inhibition of transcription or translation of exogenous viral genetic information. Very little is known of the mechanisms of the non-antiviral effects. The question has been raised whether or not these effects are interdependent at some level of the induction. It has been proposed (15) that the cell membrane might be a candidate for a common target which, influenced by the interferon, acts as a trigger which initiates a series of biological responses.

Various consequences of the interferon interaction with cells, observed in different laboratories, have been interpreted to be attributable to alteration(s) of the cell membrane (1 3 14 15 22). In the present communication we report some functional and scanning electronmicroscopical observations

of interferon effect on uninfected and virus infected human amnion cells, with special reference to the cell membrane.

MATERIALS AND METHODS

Cells The U-line of human amnion cells was received from Dr *K. Cantell*, Helsinki. It was grown in Eagle's Minimal Essential Medium (MEM) with addition of 5 per cent calf serum, NaHCO and antibiotics. The cells were maintained in the same medium with 2 per cent calf serum. The same serum concentration was used in all experiments in this study

Human embryonic lung cells (HEL) were prepared in this laboratory They were grown in 50 per cent each of MEM and medium 199 with addition of 10 per cent calf serum, $NaHCO_3$ and antibiotics. The serum concentration was reduced to 5 and later to 2 per cent during maintenance. HEL cells were used between their 5th and 15th passage.

Virus Vesicular stomatitis virus (VSV) Indiana strain, was grown in 2 days old cultures of L-F mouse fibroblast cells. When the cytopathogenic effect was complete, usually after 2 days, the culture fluid was collected after freezing and thawing. Virus was stored at –70 C, and a fresh ampoule was used in each experiment. Infectivity titres were assayed by the end point micromethod on L-F and on U cells.

Interferon Human interferon was produced in leukocytes, according to *Cantell et al* (5) The supernatants of the Sendai-virus-induced leukocytes were characterized as interferon by the standard criteria and were used without further purification. In some experiments, partially purified leukocyt interferon preparations, given to us by Dr *K. Cantell*, were used. As the results obtained with this preparation were identical to those of the crude samples they are not presented separately

The antiviral activity of interferon preparations was tested by the infectivity inhibition micromethod employing HEL cells and VSV as described in detail elsewhere (6) The titres were adjusted to the international standard preparation 69/19

The interferon effect on cell growth was tested as described elsewhere (7) Briefly aliquots of 10^5 U cells were seeded into at least 5 tubes per sample in 1 ml medium, containing dilutions of interferon. Control tubes contained the same medium, but no interferon. The cells were incubated stationary in 5 per cent CO atmosphere at 37 C. After 3 days, incubation, the monolayers were trypsine-versene treated and the cells were counted in haemocytometer

Assay of cell membrane integrity The release of soluble radioactive substances into the medium has earlier been used as indicator of damage to the cell membrane (23) Essentially the same method was used in the present study Tubes were seeded with 10^5 U cells each. Three days old monolayers were incubated for 2 hours at 37 C in medium containing 1 µCi of ^{3}H-uridine per ml. The cells were then washed thrice with fresh medium to remove extracellular radioactivity Interferon dilutions in MEM were added to 6–8 cultures each. Control tubes contained MEM alone. After incubation at 37 C for various times, 0.1 ml of the supernatant was removed and transferred to a scintillation vial containing 10 ml of Aerosol. Samples were counted in a Packard liquid scintillator for 10 minutes. The spontaneous release from the control cultures was considered as 100 per cent and release from the interferon treated cultures were related to the controls.

The cells and the remaining medium in some experiments were further treated with 0.1 ml of the non-ionic detergent Triton-X 100 to a final concentration of 0 25 per cent (v/v) The tubes were agitated in a Vortex mixer and incubated at 37 C for 15 minutes. This treatment causes complete lysis of the cytoplasmic membrane releasing all radioactivity into the medium. No intact cells could be seen by lightmicroscopic examination. One tenth of 1 ml of the lysate was transferred to a scintillation vial and counted, as described above.

Preparation of lls for scanning electronmicroscopic examination 10^4 U-cells were seeded into flat bottomed plastic trays, containing flying cover slip in 1 ml MEM, containing various interferon concentrations. The cultures were incubated in 5 per cent CO atmosphere at 37 C for 24 or 72 hours. No toxic effect on any of the interferon treated cultures could be observed by light microscopy

The specimens were fixed in 2.5 per cent glutar aldehyde in 0 1 M phosphate buffer (pH 7 4) for 2 hours and postfixed in osmium tetroxide in Tyrode's sol tion (pH 7 4) for 1 hour The specimens were then dehydrated in graded ethanol solutions and dried by the critical point method in fluid CO (Sorval critical point drying system) The mounted specimens were then coated with a thin layer of carbon and gold-palladium in an Edwards vacuum coating unit and examined in a Jeol JSM scanning electron microscope.

RESULTS

Antiviral Activity of Interferon in U Cells

It is known that U cells are highly sensitive for antiviral activity of interferon. These cells are therefore routinely employed in assays of antiviral activities. The titre of interferon in

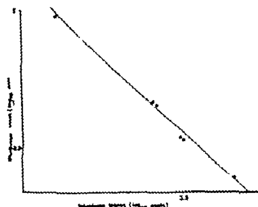


Fig. 1 Correlation between the antiviral titre of interferon and the titre of challenge virus as measured by the *infectivity inhibition microassay*

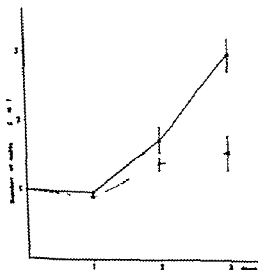


Fig. 2 Growth of U-amnion cells in culture with and without 100 units interferon per ml medium. 10^5 cells were seeded in each tube. Interferon was added simultaneously. Five interferon treated cultures □——□ and *In* controls ●——● were trypsinized and counted each day. Vertical lines indicate 1 S.D.

logarithmic units, tested in a micro-infectivity inhibition method is correlated along a straight line to the dose of infecting VSV (Fig. 1).

Treatment of U cells with 100 units of interferon 6 hours prior to inoculation with 10 $TCID_{50}$ VSV reduced the titre of infectious virus after 18 hours incubation by more than 2 log_{10}. Extracellular and intracellular virus titres were reduced to the same extent.

Growth Inhibitory Activity of Interferon on U Cells

The growth rate of U cells was tested in the continuous presence of 100 units per ml of interferon. Six tubes were trypsinized each day together with 6 control tubes, and the number of cells was counted. Interferon treated cells grow more slowly than the control cells (Fig. 2). The differences were significant on the third day. This growth inhibitory effect was dose dependent, thus 10 units interferon per ml reduced the cell numbers by ca 15 per cent, 100 units by practically 50 per cent, counted after incubation for 3 days.

Interferon Inhibition of Spontaneous Release of Radioactivity from Uninfected U Cells

After uptake of ^{3}H uridine into the cultured U cells, some of the radioactivity is



Fig. 3 Spontaneous release of ^{3}H-uridine from cultured U-amnion cells into the medium. Cells were cultured for 2 hours in medium containing 1 µCi ^{3}H-uridine per ml. After incubation the cells were washed thrice and supplied with fresh medium. Samples were removed at the indicated times and the released radioactivity was estimated. Other tubes were treated identically except that the medium was changed every 15th minutes following removal of the radioactive [illegible]. Columns show release during the last 15 minutes, line shows total released radioactivity

TABLE 1 *Distribution of Radioactivity between Supernatant and Cells in Interferon Treated and Control U-amnion Cell Cultures*

Cells	Total radioactivity (lysed cells + supernatant)	Released radioactivity (supernatant)	
	CPM	CPM	Per cent of total
Control cells	834	191	23
Cells treated with 100 unit IF	931	106	11

Cells were cultured in medium containing 1 μCi ^{3}H-uridine per ml. After 2 hours incubation the cells were washed 3 times and supplied with 1 ml fresh medium containing 100 units of interferon. Control cells did not receive interferon. After 30 minutes incubation supernatants and lysed cells were prepared as described under Methods.

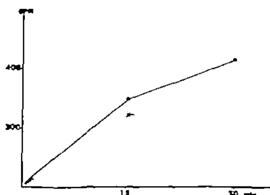


Fig 4 Influence of 200 units interferon □——□ on spontaneous release of radioactivity from cultured U-amnion cells. Interferon was added immediately after removal of radioactive medium. Experimental details as described in Fig. 3.

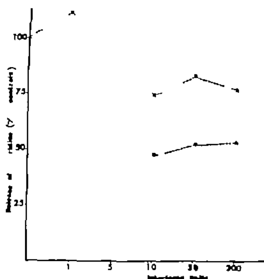


Fig 5 Influence of various doses of interferon on spontaneous release of radioactivity from cultured U-amnion cells. Various concentrations of interferon were added to the cultures immediately after removal of radioactive medium. Samples were removed at 15 minutes (closed circles) and at 30 minutes (closed squares). The counts in the interferon treated cultures were related to controls without interferon (100 per cent)

released spontaneously into the medium (Fig 3) The rate of release is maximal immediately after removal of the H uridine-containing medium. If the cell mebrane is damaged the leakage increases (23) On the assumption that interferon affects the cell membrane we have tested whether interferon treatment influences the rate of spontaneous release of a soluble radioactive substance. Interferon, 200 units per ml, diluted in MEM was added to the cells immediately after removal of H-uridine containing medium. Control tubes contained the same medium but no interferon. One tenth of the supernatant was removed after incubation for 15 and after 30 minutes and the radioactivity was assayed. The supernatant of inter

Fig. 6 Scanning electron micrograph of untreated U-line human amnion cells. Whereas most of the cells are polygonal of shape and somewhat flattened on the substrate some spherical cells are also present. Note the numerous slender cytoplasmic extensions (microvilli) Some of the round cells show blebs.



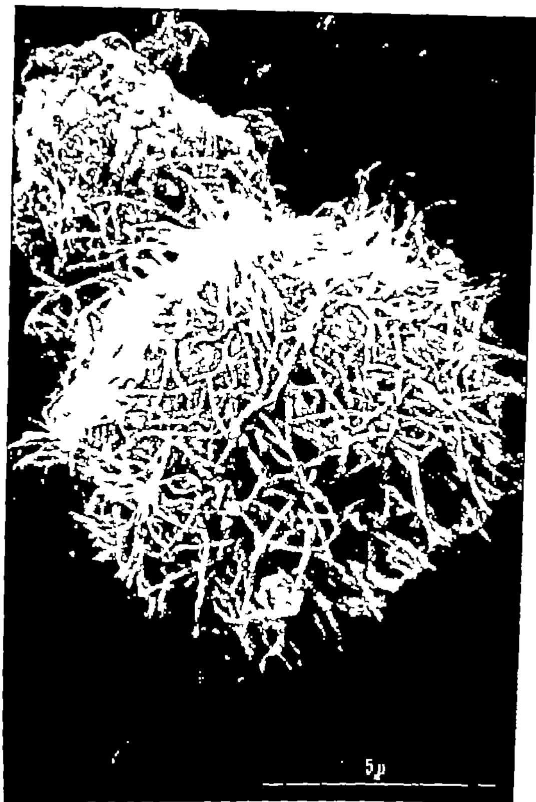

5 μ



Fig 6 Control cells. Detail of a spherical cell with numerous blebs and some microvilli.

← *Fig 7* Control cells. Detail of a spherical cell with numerous microvilli


10μ

feron treated cultures contained less radioactivity indicating a reduced release (Fig. 4). The differences were reproducible and significant. A control experiment indicated that the remaining radioactivity approximately 80 per cent in the case of control cells and 90 per cent in the interferon treated cells, was associated with the cells (Table 1). The inhibitory effect of interferon on the spontaneous release of radioactive substance was dose-dependent, up to 10 units interferon per ml. Further increase of concentrations did not enhance the effect (Fig. 5). The effect seems to be maximal during the initial period. Longer incubation period seems to reduce the differences between total release from interferon treated cells and control cells.

Scanning Electron Microscopic Observations

Control cells. Control cells, as well as the interferon treated cells were cultured under conditions as identical as possible. The cells were not removed from the coverslip (on which they had been growing) during the preparation procedure for scanning electron microscopy. As shown on Fig. 6, most cells were partly spread on the surface of the glass, but with a rounded shape in the region containing the nucleus. Long, flattened pseudopodia were often seen. On the surface, numerous slender cytoplasmic projections with a diameter of about 0.1 micron were found. Some of them appeared to be bridging neighbouring cells (Figs. 6–8).

Rounded-up cell forms were also seen (Fig. 6). These were covered either with numerous microvilli (Fig. 7) or with a mixture of microvilli and cytoplasmic blebs (Fig. 8).

After 72 hours incubation the number of cytoplasmic projections appeared to be slightly reduced as compared with the specimens incubated for 24 hours.

Interferon treated cells. In cell cultures treated with the different concentrations of interferon, no clear-cut differences could be demonstrated between the cells treated with different concentrations or without interferon. The growth pattern, the cell shape and surface with the numerous microvilli or blebs corresponded to those seen in the control specimens (Figs. 9 and 10).

DISCUSSION

Our results confirm the earlier observations that interferon has profound effects on the homologous cells, effects which can be registered by a number of different biological parameters. Comparable amounts of interferon reduced the replication of VSV and the cytopathogenic effect caused by it, reduced the growth rate of uninfected cells, and reduced the spontaneous release of ^{3}H-uridine from the cells.

Under the experimental conditions employed in this study interferon did not influence the process of virus release from the infected cells, but release of radioactive substances from uninfected cells was reduced. Reports from several laboratories indicate that, in some cases, especially those of oncogenic RNA viruses, a late step in the multiplication, maturation or release is affected by interferon (1, 9). One of the alternative hypotheses forwarded is that interferon or the antiviral protein might interact with the cell membrane to inhibit virus release (1). Our results may support the notion of membrane stabilization by interferon. It may also be recalled, that there is no convincing evidence that interferon actually passes beyond the cell membrane (10).

To our knowledge, morphological alterations of the cell surface as a result of interferon treatment have not been reported, but none of the earlier observations included scanning electron microscopy. Our scanning electron microscopic studies revealed surface characteristics of both the controls and the interferon treated cells with numerous microvilli and rounded cells with blebs. Such blebs

Fig. 9. U cells treated with interferon, 200 units per ml for 24 hours. Both flattened and spherical cell types are present. The rounded cells contain microvilli as well as blebs.

2*


10μ

are common on cells in mitosis (24) and it may be suggested that the rounded cells are in an early phase of mitosis. There was, however, no clear difference in surface appearance of the interferon treated U cells and controls, even when the concentration of interferon was as high as 2000 units per ml, many times more than necessary to produce numerous functional alterations. Functional alterations can, of course be present without interferring with the surface appearance and structures like microvilli and blebs formation. We believed that some data indirectly suggested that such changes might occur. Dibutyryl cyclic AMP treatment caused regression of the numerous long cell surface microvilli present on the L 929 cells (26). Furthermore, several reports show that there is a relationship betwen the interferon system and cyclic AMP. Interferon treatment increases the cellular cyclic AMP levels both *in vitro* and *in vivo* (4, 25). The interrelationship between cyclic AMP and interferon seems to vary in different cells, even in the same type of cells of different ages (19). It is possible that, under different experimental conditions or in another cell type, the interferon treatment could produce visible changes, although mouse interferon treatment of L-F_1 fibroblast cells under the same conditions as in this study did not result in morphological changes that could be observed by light microscopy and scanning electron microscopy (*Degré & Hovig* unpublished observations).

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Fig. 10. Detail of interferon treated cell with both microvilli and blebs.

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STAPHYLOCOCCUS AUREUS STRAINS ISOLATED IN DANISH HOSPITALS FROM JANUARY 1st, 1966 TO DECEMBER 31st, 1974

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Rosendal, K., Bülow P Bentzon, M. W & Eriksen, K. R. *Staphylococcus aureus* strains isolated in Danish hospitals from January 1st, 1966, to December 31st, 1974 Acta path. microbiol scand. Sect. B, 84 359–368, 1976

During the years 1966–1974 167,297 strains isolated from 167,297 patients or staff members in Danish hospitals were registered at Statens Seruminstitut. All the strains were phage-typed and examined for production of a 'Tween-80-splitting enzyme and resistance to mercuric chloride. 158,236 strains were examined for resistance to antibiotics. Since 1968, a steep decrease in the number of strains resistant to three or more antibiotics (multiple-resistant) and in strains of the 83A complex was noticed. In recent years an increase in strains belonging to phage-group 1 and in those referred to as miscellaneous and non-identified was registered. The increase in the non-typable strains might be explained by the shift of the concentration of the typing phages from 1000 × RTD to 100 × RTD It is concluded that at least two factors may have contributed to the reduction of the multiple-resistant strains an altered antibiotic policy restricting the use of streptomycin and tetracyclines, and an improved hospital hygiene, diminishing the spread of identical strains within the various departments. However it is emphasized that the consumption of methicillin is still increasing.

Key words *Staphylococcus aureus* strains; antibiotic resistance phage-typing Danish hospitals.

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At Statens Seruminstitut, Copenhagen, the registration of phage-typed *Staphylococcus aureus* strains from hospitals all over the country is still performed as described in publications from 1963 (20) and 1967 (16) which deal with the material from the beginning of the registration in April, 1960 until the end of December 1965

In these surveys, a steep decrease in the number of strains belonging to the 52, 52A, 80 81 complex was noticed, whereas strains belonging to the 83A, 84 85 93 89 complex (= 83A complex) and especially those resistant to three or more antibiotics were registered with increasing frequency

The present survey deals with the material collected over a nine year period ending December 31 1974 It was found that the changes described previously continued dur

TABLE 1 *The Material*

Year	1966	1967	1968	1969	1970	1971	1972	1973	1974	Total
Number	15682	16044	16235	18626	19305	18395	20002	21303	21707	167297
Examined for resistance to antibiotics	14712	14990	15111	17604	18393	17478	19024	20254	20670	158236
TW— %	34	36	36	35	33	29	27	22	19	
Hg+ %	36	35	32	29	26	21	18	13	11	

TW— = do not produce the Tween-80-splitting enzyme.
Hg+ = resistant to mercuric chloride.

ing the first three years and that from then on a decrease in the multiple-resistant strains was observed.

Since 1966 it has been possible to record the occurrence of the methicillin-resistant strains. Furthermore, evolutionary events within the 83A complex have been followed during recent years, as it has been possible to perform a subdivision of the complex by using the new international typing phages 84 85 and 89

MATERIALS AND METHODS

The Material

A "Total material" (Table 1) comprises all phage-typed *Staphylococcus aureus* strains isolated from persons connected with Danish hospitals, patients and staff members. Only one strain per person is included, selected as previously described (20)

B Bacteraemia strains. For comparison, some figures from a material consisting of bacteraemia strains only (19) have been made use of

The methods are mainly those described in papers from 1963 (20) and 1967 (16)

The alterations are as follows

Registration

The information from the material from 1966 was extracted from the original cards. The results from 1967–1974 were transferred to punch-cards, using special cipher-codes for the registration of phage-types/groups, sources, resistance to antibiotics and *mercuric chloride and production of a* Tween-80-splitting enzyme.

Bacteriophage-typing

New typing phages has been added to the basic set (2) in 1966 (phage 84 and 85) 1969 (phage 89) (17) and in 1973 (phage 94) (3) The strains typed by phage 94 have been recorded as "miscellaneous" The phage previously referred to as "6357" (5) has been internationally designated as phage 93.

From June 1st, 1971 the strains non-typable at RTD (routine test dilution) have been retested with the phages in a concentration of 100 × RTD instead of 1000 × RTD In order to examine the effect of this change, freeze dried bacteraemia strains, non-typable at 100 × RTD were retyped, using the concentration of 1000 × RTD (Table 2)

RESULTS

From Table 1 it is seen that the material consists of 167,297 strains (isolated from the same number of persons) most of which have been examined for resistance to antibiotics. During the whole period the number of strains has been steadily increasing

Changes in the Properties of the Strains

Resistance to antibiotics (Fig 1) The percentage of strains sensitive to all antibiotics and resistant to penicillin (P) has been fairly constant, but strains resistant to the other antibiotics tested (streptomycin (S) tetracyclines (T) chloramphenicol (C) erythromycin (E) and methicillin (M)) have since 1968 become less frequent. It is remarkable that the declining parts of the curves representing S and T resistance run parallel.

The changes of resistance are further analysed in Fig 1 B which shows a decrease in multiple-resistant strains (resistant to three or more antibiotics) from 25 per cent in 1967 to 7 in 1974 and an increase in strains resistant

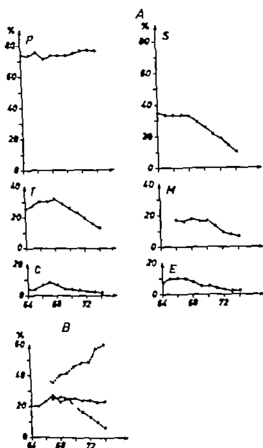


Fig 1 Antibiotic sensitivity of the strains (per centage) The antibiotic sensitivity of strains from a previously published material (16) from the years 1964–1965 is also shown in this figure.

A. Strains resistant to:

P = penicillin	S = streptomycin
T = tetracyclines;	M = methicillin
C = chloramphenicol;	E = erythromycin.

B. ●- - -● Strains resistant to P and sensitive to other antibiotics investigated.
●—·—● Strains resistant to P + S + T ± other antibiotics as well.
●——● Strains sensitive to all antibiotics.

to P only from 36 per cent to 60 over the same period.

Phage-types/groups The percentage oc currences of the various phage-groups are shown in Figs. 2 and 3. The major changes are strains of the 52, 52A, 80 81 complex are still decreasing strains of the 83A, 84 85 93 89 (83A) complex reached a peak in 1969–1970 whereafter the percentage fell from 25 to 14 in 1974 and a considerable increase of non-typable strains was noticed from 1971

Further investigation of the 83A complex (Fig 3) showed that strains lysed by all five typing phages have almost disappeared, whereas strains sensitive to phage 83A plus some of the other phages and types 84 and 85 are increasing. Types 89 and 84/85/93/89 have decreased since 1969

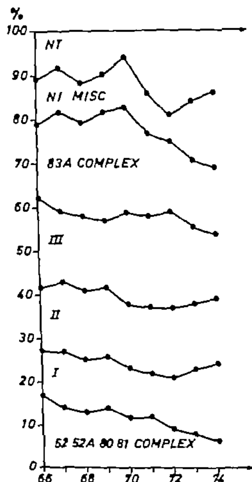


Fig 2 Percentage contribution of the various phage-groups/complexes. Cumulative curves
NI = Non-identified
Misc. = Miscellaneous
NT = Non-typable

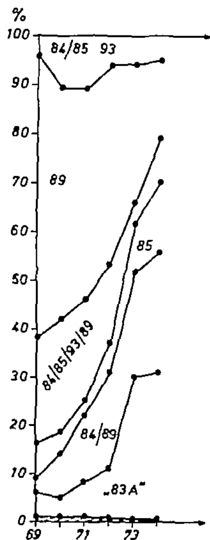


Fig 3 83A complex. Cumulative curves.

84/(89) = Strains lysed by phage 84 whether or not in combination with phage 89.

"83A = Strains lysed by phage 83A either solely or in combination with some, but never all, of the phages 84 85 89 and 93

/////// = Strains lysed by all five typing phages in question.

As the increase in the non-typable strains coincided with the change of phage concentration from 1000 × RTD to 100 × RTD bacteraemia strains from recent years were tested with concentrations at 1000 × RTD which led to a reduction of non-typable strains to a percentage of 6 equal to that of previous materials (Table 2)

Resistance to mercuric chloride and production of the Tween-80-splitting enzyme The percentages of strains resistant to mercuric chloride (Hg+) and those not producing the Tween-80-splitting enzyme (TW—) have decreased since 1968 and 1971 respectively (Table 1)

Correlation of the Various Properties of the Strains

Resistance to antibiotics and phage-type/group In Table 3 the material from 1974 is compared with that from 1969 in which a more detailed subdivision of the 83A complex became possible. The previously described prevalence of certain antibiotic patterns within certain phage-groups (16) is also found in the present material. The highest percentage of strains resistant to P+S is still seen within the 52, 52A, 80, 81 complex, and that of multiple-resistant strains among strains of the 83A complex.

However the changes in antibiotic resistance found in the material as a whole are also found within the various groups a higher proportion of strains are resistant to P only and the multiple resistant strains have become fewer among strains of the 83A complex, the multiple-resistant strains have decreased from 70 per cent in 1969 to 38 in 1974

These changes are to a certain degree caused by variations in the proportion of the

TABLE 2. *Percentages of Non-typable Strains among Bacteraemia Strains*

Years	NT at 100×RTD	NT at 1000×RTD	Total number of strains
1971	21	10	329
1972	18	5	577
1973	13	5	611
1974	12	4	516
Total	16	6	2033

TABLE 5 *Correlation of Various Properties of the Strains*

	Per cent sensitive to antibiotics		Resistant to (per cent): P		PS		PST + PSTE + PSTEM + PSTM		Per cent of the complex in question		Total number	
	1969	1974	1969	1974	1969	1974	1969	1974	1969	1974	1969	1974
Phage-type/group/complex												
52, 52A, 80, 81 complex	28	40	24	32	24	12	16	7	–	–	2490	1573
Type 80/81	4	13	21	25	39	39	25	13	15	5	382	73
Type 52/52A/80/81	29	40	23	52	27	15	15	7	57	73	1407	1004
Other types of the complex	39	44	30	33	9	4	12	6	28	22	701	296
Group I	42	32	51	63	2	1	2	1	–	–	2095	3776
Group II	33	26	60	67	1	1	1	1	–	–	2802	3189
Group III	18	14	60	71	2	1	11	4	–	–	3212	3327
83A complex	4	8	˚70	48	1	1	70	38	–	–	4412	3005
Type "83A	15	9	68	70	1	2	8	14	5	30	223	889
Type 84/(89)	11	11	45	16	2	1	57	67	3	25	121	747
Type 89	2	5	3	13	1	1	91	76	56	16	2468	487
Other types of the complex	6	8	37	63	1	1	49	16	36	29	1600	882
Per cent among strains of the antibiotic pattern												
TW–	16	15	20	16	27	19	83	56				
Hg +	4	5	7	4	61	34	91	80				

For an explanation of the nomenclature, see Fig. 3

TABLE 4 *Correlation of Various Properties of Multiple-resistant* Strains*

	Per cent				Per cent of *multiple-resistant*	
	TW–		Hg+			
	1969	*1974*	*1969*	*1974*	1969	1974
Phage-type/group						
Group III	73	44	81	72	9	8
83A complex	94	63	96	90	76	72
Type "83A	(44)	16	(83)	87	1	8
Type 84/(89)	73	66	89	90	1	32
Type 89	97	74	97	93	35	24
Other strains	35	29	73	47	15	20
Total	83	56	91	80		
Number of strains					4086	1491

* Multiple-resistant = resistant to PST PSTE, PSTEM and PSTML
The brackets indicate that the figures are too small to permit a calculation.
For an explanation of the nomenclature, see Fig. 3

various phage-*types* (Table 3 column 4) whose antibiotic resistance is fairly stable. For *instance, the resistance of the 83A complex is* influenced by the increase of type "83A" of which 70 per cent is resistant to P only Yet, *strains resistant to* P+S have become less frequent in type 52/52A/80/81 and the percentage of multiple-resistant strains has increased in type 84 and decreased in type 89

Resistance to antibiotics and occurrence of Hg+ and TW– strains The highest proportion of Hg+ and TW– strains is found among multiple resistant strains, even though the percentage of the TW– strains shows a decreasing tendency (Table 3)

Phage pattern and occurrence of Hg+ and TW– strains The properties of the multiple resistant strains are further examined in Table 4 The percentage of TW– strains has decreased in all phage-groups. The percentage of Hg+ strains does not vary much for the two years registered except among strains referred to as "other" the percentage composition of which does not differ for the years in question.

As a rule, resistance to mercuric chloride and Tween negativity *cannot* be correlated to phage-pattern. However there are a few exceptions the percentage of TW– strains is higher among strains of phage-type 80 and 71 and lower in strains of phage-type "83A" than among other strains of the same antibiotic pattern within the same phage-group Furthermore type 80/81 includes a higher proportion of Hg+ strains than do other strains of the 52, 52A, 80 81 complex.

Clinical Sources and Distribution of Phage-groups/Complexes

As the contribution of strains from most sources was found not to vary much from year to year only the percentages from 1974 are given in the upper part of Table 5 However there is an increase in strains isolated from cicatrices and wounds (Table 5 lower part)

The percentage occurrence of some phage groups/complexes among strains from some sources is given in Table 6 In nearly all instances, strains of the 52, 52A, 80, 81 and 83A complexes have decreased since 1967 Group III strains were more frequently isolated in 1970 than in 1967 but have also decreased during the last four-year period.

TABLE 5. *Clinical Sources of Strains. Percentage of Total Material*

Source	1974 Per cent
Abscesses	8
Pustule	5
Eye	1
Ear	7
Nose	5
Blood	3
Fistula	2
Urine	10
Burns	2

Source	1967 Per cent	1974 Per cent
Cicatrices and wounds	12	21
Not specified	32	28

Yet in strains from wounds group III is now the most frequently found phage-group.

The percentages of the M resistant strains, also given in Table 6, have decreased since 1967 for some of the sources, however the highest percentages are found in 1970

Comparison with Bacteraemia Strains

It is not possible to state whether the strains of the present material were isolated from processes originating before or after hospitalization. However it is possible to compare properties of these strains with those of strains from bacteraemia cases, where hospital strains can be separated from other strains.

Table 7 shows that strains from the "Total material" occupy an intermediate position between strains of known hospital origin and strains originated outside hospitals, which indicates that the "Total material"—as might be expected—contains strains from hospital cases as well as from outside cases.

DISCUSSION

The changes in antibiotic resistance described here are similar to those observed by authors from various countries (13 22, 13 14) dating back to 1959 (4)

These results may give rise to the questions of why certain strains become endemic, and what properties are able to give them advantages over other strains under certain circumstances.

In the Danish material, multiple-resistant strains of the 83A complex are being replaced by strains resistant to P only which do not belong to the genetically related group III but mainly to unrelated groups.

It is possible that strains resistant to a limited number of antibiotics may in certain respects be superior to multiple resistant strains results recently published by *Lacey & Chopra* (10) showed that loss of resistant markers gave the strain an advantage over the multiple-resistant parent strain. If this example can be generally applied, it supports the theory that multiple-resistant strains are superior to

TABLE 6. *Percentage Occurrence of Methicillin Resistant Strains and of some Phage Groups/Complexes among Strains from Various Clinical Sources from the Years 1967 1970 and 1974*

Source	M-resistant strains			52 52A, 80, 81 complex			Group III			83A complex			Non-typable strains		
	1967	1970	1974	1967	1970	1974	1967	1970	1974	1967	1970	1974	1967	1970	19
Abscesses	2	3	1	31	23	11	9	16	10	9	11	9	6	5	1
Cicatrices	16	19	4	13	12	7	16	20	13	34	32	15	7	5	
Wounds	7	11	2	11	10	5	19	25	18	28	5	15	7	5	1
Trachea	21	18	4	9	9	5	14	21	15	31	28	15	13	5	1
Sputum	14	22	8	8	9	5	16	21	16	33	32	19	8	5	1
Urine	23	20	11	7	9	5	19	21	21	45	43	26	10	5	1

TABLE 7 *Various Properties of Strains of Different Origin. Strains from Total Material Compared with Bacteraemia Strains*

Year	Res. to P only per cent			Res. to T per cent			Res. to M per cent			83A complex per cent		
	Hos	*TM*	*OH*	*Hos*	*TM*	*OH*	*Hos*	*TM*	*OH*	*Hos*	*TM*	*OH*
1967	23	36	47	60	30	17	46	16	5	53	23	10
1970	31	46	47	50	25	18	40	17	8	46	24	18
1974	56	60	62	22	13	9	15	6	3	26	14	14

Hos = Bacteraemia strains from cases of hospital origin.
OH = Bacteraemia strains from cases originated outside hospitals.
TM = Total Material.

less resistant strains only when given protection by an antibiotic pressure.

However a correlation between the decline of M resistant strains and the diminished use of methicillin has not been demonstrated, neither in four large Danish hospitals (19) nor in any other country (11 23 8) On the other hand a correlation between the proportion of resistant strains and the reduced use of streptomycin and tetracyclines has been found both here and elsewhere (19 14)

Yet, even if it is accepted that the altered antibiotic policy at least partly explains the increase in strains resistant to P only the shift to strains of group I and miscellaneous strains is not accounted for These strains must possess properties enabling them to outgrow other strains under the present circumstances.

Several authors (4 11 23) have suggested that improved hygienic measures may also contribute to the reduction of the multiple-resistant strains through a decrease of the cross-infection rate The present authors investigated the spread of identical strains within larger hospital departments in the years 1969 and 1974 (19) As expected, the multiple-resistant strains from 1969 had been replaced by strains resistant to P only Furthermore, the number of cases caused by one strain was modest in 1974 compared to the number in 1969 but experience rather than statistical evidence, told that the P resistant strains actually began to spread when they had been introduced into the hospitals. Thus, an improved hospital hygiene cannot alone account for the changes observed, and it is probable that the explanation is more complex.

The suggestion forwarded by *Pulverer & Damen* (15) and *Otaya* (12) that "newer" antibiotics, *e g* the cephalosporins, might be able to eradicate the multiple-resistant strains, cannot explain the changes found in the Danish material, as in Denmark the consumption of these drugs as compared to that of methicillin is insignificant.

The mechanisms causing changes of resistance patterns in staphylococci are known to be mutation and transduction (see 9) whereas lysogenization which does influence the phage pattern, has not previously been shown to affect antibiotic resistance. Recently however *Blackwell & Feingold* (1) found that loss of the prophage(s) blocking lipase production was combined with loss of certain resistance determinants as well. In the Danish material more of the multiple-resistant strains have become TW + thus showing loss of the prophage(s) blocking the Tween-80-splitting enzyme. These strains are assumed to be genetically unstable, owing to their abundant content of DNA elements, and the possibility cannot be excluded that the TW + variants are the more stable descendants lacking some of the original resistance markers. Furthermore, the S and T determinants seem to be lost simultaneously (Fig 1) These observa-

tions seem to be in agreement with the experimental data obtained by *Bülow* (6) suggesting that resistance markers to S and T are linked and can be transduced by a phage blocking the above mentioned enzyme.

The changes within the 52 52A, 80 81 complex with shift from type 80/81 to type 52/52A/80/81 also indicate phage activity influencing the phage type (21) A preliminary investigation of the strains of the 83A complex has indicated that similar events may take place here these findings will be published separately (18)

The strains non-typable at 100 × RTD do not represent a homogeneous group Retyping at 1000 × RTD disclosed that their distribution corresponded exactly to that of the whole material, and that their increase was due to the change in concentration of the phage suspensions. However it is noteworthy that the percentage of strains non-typable at RTD has been constant throughout the whole period investigated.

The changes of the properties registered here are presumed to influence the outcome of staphylococcal septicaemia cases from the same years (7) During this period, a slight decrease in mortality was seen, which was most probably due to better facilities for adequate antibiotic treatment.

At the same time, another improvement was noticed the spread of identical strains within hospitals was diminished.

Thus in Denmark at present two factors may have contributed to the more favourable situation a more restricted use of streptomycin and tetracyclines, and an improved hospital hygiene. But the equilibrium may easily be disturbed, and it is to be hoped that future developments will continue along the same lines.

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EFFECT OF ANTIBIOTICS ON INTERFERON PRODUCTION IN MICE

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Rollag, jr. H. & Degré M. Effect of antibiotics on interferon production in mice. Acta path. microbiol. scand. Sect. B, 84: 369-372, 1976.

Neomycin, chloramphenicol, gentamycin, rifampicin and oxytetracycline were tested for their influence on virusinduced interferon (IF) production in mice. Repeated intraperitoneal (i.p.) injections of antibiotics were given to produce significant serum concentrations during the time of IF production, induced by i.p. injections of Newcastle disease virus (NDV). IF titres in antibiotic-treated mice determined 6 hours after induction were compared to titres in control mice given NDV only. The IF production was not significantly modified in most of the antibiotic treated mice. Only the highest dose of chloramphenicol (2500 μg/mouse) appeared to cause a reduction in IF production ($p<0.10$). Addition of antibiotics in vitro did not alter the antiviral titres of IF.

Key words: Interferon production; effect of antibiotics; mice.

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Differential diagnoses between viral and bacterial infections may be extremely difficult to establish on the basis of clinical findings. Antibiotics are widely employed, often on a weak indication, before the presence of causative bacteria has been established.

It is often argued that antibiotic treatment, even if given unnecessarily, at least does not harm. This argument of course must be strongly qualified. Thus selection of resistant strains of bacteria is a real and unwarranted possibility, and toxic side effects of some antibacterial drugs are also known.

We were interested in possible side effects on the interferon (IF) system, one of the early determinants of the progress and development of viral infections. Interferon production requires *de novo* synthesis of RNA and protein. Metabolic inhibitors, e.g. actinomycin D, puromycin, p-fluorophenylalanin and cycloheximide, which interfere with these processes, inhibit IF production (9). It is also known that some antibiotics although to a minor extent may affect metabolic processes in eucaryotic cells (4, 5, 6, 7, 8). However, very little information is available whether there is an interaction between the interferon system and antibiotics. In the present communication we report some observations on IF production *in vivo* in the presence of five frequently employed antibiotics.

MATERIALS AND METHODS

Antibiotics. The drugs and th[...] [...]ores injected intraperitoneally to Ham, ICR C[...]/Bom albino mice

are listed in Table 1 Rifampicin was dissolved and diluted in HCl pH 2.5 the other drugs were dissolved and diluted in distilled water

Assay of antibiotic activity The mice were bled from the tail tip immediately before the 2 and 3 injection and at the termination of experiment, and the concentrations of the antibiotics were determined. The antibiotic activity of rifampicin, oxytetracycline and gentamycin was determined by the micromethod of "AB Biodisk" Stockholm (3) We were not in possession of a suitable micromethod for determination of the activity of neomycin and chloramphenicol.

Viruses Newcastle disease virus (NDV) was propagated in embryonated hens eggs. The allantoic fluid was harvested after 3 days of incubation at 37 C and tested for haemagglutinating activity on guinea pig erythrocytes. Samples with haemaggl tinating titres of 256 or more were pooled and kept at –20° C.

Vesicular stomatitis virus (VSV) Indiana strain, was propagated in the allantoic cavity of embryonated hens' eggs, and two passages in L-F1 mouse fibroblast cells. When the cytopathogenetic effect was complete, usually after two days of incubation, the cultures were frozen and thawed twice and the supernatant was collected. The samples were titrated for infectivity by the end point micromethod on L-F1 cells and kept at –20 C.

Influence of antibiotics on interferon production Antibiotics were injected i.p. 4 times with 2 hours interval, each dose into 5 mice. Two hours after the first injection the mice were given 0.2 ml *NVD HA titre 256* by the i.p route of administration. Controls were given NDV only Six hours after the induction with NDV the mice were exsanguinated and IF titres in the pooled sera were determined. Each experiment was repeated 2–3 times, and each pool was titrated twice.

Assay of interferon activity Mice were exsanguinated 6 hours after induction with NDV Blood from 5 mice was pooled. The serum was separated after 1 hour t room temperature and at 4 C overnight. The serum was then adjusted to pH 2 by 1N HCl to destroy infective irus. After two days at 4 C the serum was neutralized by 1N NaOH. IF activity was assayed by an infectivity inhibition micromethod that employs L-F1 fibroblast cells and VSV (2) A standard mouse interferon produced in the laboratory equivalent to 2.2 units (National Institutes of Health) assayed by the micro infectivity inhibition test, was included in each assay

RESULTS

Administration of antibiotics The maximal doses of the antibiotics tolerated by mice were determined by toxicity studies. Increasing

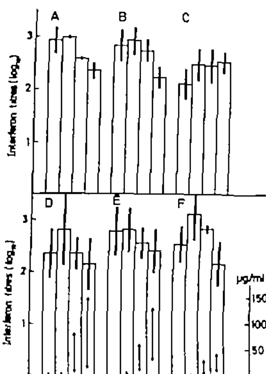


Fig 1 Interferon in mouse serum induced with NDV in the presence of various concentrations of (A) cycloheximide (single dose) and antibiotics (repeated doses) (B.chloramphenicol, C:neomycin, D gentamycin, E.rifampicin, F:oxytetracycline) *The left-side column in each group represents* control mice. The employed concentrations are listed in Table 1 Vertical bars indicate 1 S.D The right-side scale and the arrows inside the columns (D,E,F) show the range of antibiotic concentrations in the serum during the course of experiments with the given concentrations.

doses of antibiotics were injected i.p. into three mice each. The injections were repeated 4 times with two hours interval. Mice were observed and the mortality was recorded. The highest doses tolerated and the other doses employed in the following experiments are listed in Table 1

The range of the concentrations in the serum of the various antibiotics during the course of the experiments is indicated in Fig 1

Influence of antibiotics on interferon production The results of these experiments are summarized in Fig 1

The titres in parallel experiments varied

TABLE 1 *Drugs Trade Name and Doses of the Drugs Given i.p. to Mice*

Drug	Trade name Lot No	Doses in µg given i.p. to mice			
Cycloheximide	Cycloheximide Sigma Lot No 3202840	0	1	5	10
Chloramphenicol	Kloramfenicol Arco" Lot No 1073	0	25	250	2500*
Neomycin	Neomycin "NMD" Art. No 1M226	0	5	50	500*
Gentamycin	Garamycin "Schering Lot No AMK 495	0	50	500	5000*
Rifampicin	Rifampicin "Ciba/Geigy" Lot No 10127	0	25	250	2500*
Oxytetracycline	Terramycine Pfizer" Lot No 30151181	0	100	1000	1500*

Maximal dose tolerated.

quite considerably e.g IF levels in mice given NDV only varied from $\log_{10}$ 2.2 to $\log_{10}$ 2.8. These variations are expressed in the usable standard deviations (Fig 1)

The IF titres in the mice that received antibiotics differed only slightly from those in the controls. In neomycin *treated mice*, titres increased somewhat with increasing doses of the drug. Following injections of the other antibiotics, reduced IF titres with the highest doses were registered. However following the minimal dose, the IF titres were somewhat higher than those in the controls. Only the group which received chloramphenicol showed IF titre reduction of more than one standard deviation ($p<0.10$)

Effect of cycloheximide on interferon production Youngner *reported a marked depres*sion on IF production in cycloheximide treated mice when cycloheximide was given 1 or 2 hours before the injection of NDV (9) In our experiments, a significant decrease in the IF production was found when 5 or 10 mg/mouse of cycloheximide was injected as a single dose 2 hours before the injection of NDV (Fig. 1)

Effect of antibiotics on the antiviral titres of interferon A sample of an internal standard interferon was titrated by *the infectivity* inhibition micromethod in the presence of 100 µg/ml of the various antibiotics. The final concentrations chosen, corresponded to the maximal concentrations obtained in mice in the *in vivo* experiments. No significant alterations of the interferon titres were observed.

Effect of antibiotics on the infectivity end point titration of VSV To test whether antibiotics had *any direct influence on the virus* multiplication, an infectivity end point titration of VSV was carried out in the *presence* of 100 µg/ml of the antibiotics. The titres did not differ from the control assay

DISCUSSION

The largely negative results of our experiments were only partially expected. It is true that effects of antibiotics on metabolic processes of eucaryotic cells were only observed at concentrations much higher than those affecting procaryotic cells (4 5 6, 7 8) However rifampicin clearly reduced IF production by chick embryo cells at concentrations comparable to those observed in our mice (7) It is possible that the discrepancies are due to differences between the animal species. The different experimental conditions may be an alternative explanation. According to experiments performed in chick, mouse em-

bryo and rabbit kidney cells, neomycin markedly enchanced the induction of the IF mechanism by single and double stranded synthetic RNAs (1) A similar tendency was observed in our experiments, although the increases were not significant.

To summarize, the five antibiotics employed did not significantly influence the virus induced interferon production in mice when the drugs were given in "therapeutic doses" The possible exception is the highest dose of chloramphenicol which reduced the IF production. Unfortunately we were not able to assay the serum concentrations of chloramphenicol, thus we do not know whether we are really operating with concentrations within the therapeutic range in the human organism.

One should be careful not to generalize the results shown in one limited system. More data are necessary before the possibility of an interaction between antibiotics and the interferon system can be excluded.

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ANTIBODIES TO INFLUENZA A/SWINE-LIKE VIRUSES (Hsw1N1) IN HUMAN SERA ANTIGENIC STIMULATION AND CHANGES IN ANTIBODY STATUS

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Pyhälä, R. Antibodies to influenza A/swine-like viruses (Hsw1N1) in human sera antigenic stimulation and changes in antibody status. Acta path. microbiol. scand. Sect. B, 84 373–378, 1976.

Human sera collected at the end of 1975 were studied for HI antibodies to influenza A/Mayo Clinic/103/74 (Hsw1N1) The frequent occurrence of these antibodies in subjects born before 1930 suggested that they are present in about 25 per cent of the Finnish population. During the H3N2 epidemic in winter 1975–76 a low response of antibodies to A/swine-like viruses was recorded in about half of the influenza patients with a pre-existing titre, but not in any of the patients without pre-existing antibodies. A comparison of samples collected in 1969 and 1975 suggested that at the community level the antibodies to A/swine-like viruses have not decreased during the era of the H3N2 subtype.

Key words Influenza A (Hsw1N1) antibody status antigenic stimulation.

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Haemagglutination inhibiting (HI) antibodies to influenza A/swine-like viruses (Hsw1N1 subtype) are frequently found in sera of humans born before 1930 (5 8, 9 14 18) There is a general agreement that these antibodies originally resulted from infections with the A/swine like viruses, which are believed to have caused the 1918 pandemic of influenza. According to the principle of the "original antigenic sin" (5 7 8) subsequent infections with viruses of H0N1 and H1N1 subtypes maintained the level of anti-A/swine like antibodies in the subpopulation of subjects born before 1930. It was reported however that exposure to viruses of H2N2 and H3N2 subtypes, responsible for influenza A epidemics since 1957 did not evoke an antibody response to earlier subtypes (13 14 15 21 22) Somewhat contrary to these negative findings, a weak increase of anti-A/swine like antibodies was depicted as a response to H2N2 influenza vaccine in about half of 47 vaccinated volunteers (10) Due to the lack of a proper antigenic stimulus one would expect to find a decrease in the level of antibodies to Hsw1N1 viruses in the abovementioned subpopulation. Indeed, this was demonstrated by comparing samples of sera collected in 1958 and 196 (14)

Because of the outbreak of an Hsw1N1 infection in a military camp in New Jersey in

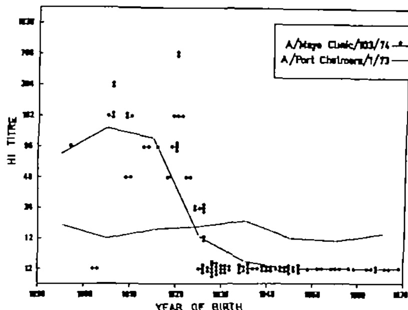


Fig. 1. HI antibodies to influenza A/Mayo Clinic/103/74 (Hsw1N1) and A/Port Chalmers/1/73 H3N2) in sera collected in autumn 1975. Geometric mean titres (GMT) connected with lines (Individual titres of <12 were regarded as 6 when calculating the GMT).

late January 1976, it became of current interest to estimate the level of immunity of populations in different parts of the world (23). As one indicator of the immunity level in Finland sera collected in autumn 1975 were tested for HI antibody to an A/swine like virus. To shed light on changes in antibody status during the era of the H3N2 subtype, another sample collected in spring 1969 was studied simultaneously. The results stimulated an analysis of heterologous antibody responses of influenza A patients in winter 1975–76.

MATERIAL AND METHODS

Three collections of sera from hospital patients sent to the Central Public Health Laboratory in Helsinki for routine virus antibody testing were studied.

1) Single sera taken in autumn 1975 from 135 patients *in the acute phase of different infectious* diseases. The sera were selected to represent subjects of all ages (age distribution in Fig. 1) and the whole country. The sera were collected before the onset of the influenza A epidemic caused by A/Victoria/75-like viruses in winter 1975–76. The sera were stored at +4 C.

2) Single sera taken in spring 1969 from 24 patients born in 1897–1921. In other respects the subjects corresponded to those in the first collection. The sera were taken after the first epidemic wave caused by influenza A strains of the H3N2 subtype. In a preliminary test the sera were studied for HI antibodies to A/Hong Kong/8/68 (H3N2) in order to shed light on the infection frequency during the epidemic of 1968–69 and thus to characterize the collection. A titre of ≥48 was recorded in 8 of the 24 subjects. This rate suggests a rate of infection of less than 33 per cent, since among old people these antibodies are known to have been more or less frequently present even before the appearance of the H3N2 subtype in 1968 (6, 9, 13, 14). The sera in the collection were stored at first at +4 C for about two years and then, after checking that they were not visibly infected, at −20° C till tested in spring 1976.

3) Paired sera obtained in winter 1975–76 from 34 patients with influenza A infection. The infection was confirmed serologically by a four fold or greater rise in complement-fixing (CF) antibodies (2) to S antigen prepared from influenza A/Finland/2/71 (H3N2). The patients were selected to fall into two categories when grouped by age: Group A: 20 subjects born in 1899–1918, before or at the beginning of the era of the Hsw1N1 subtype. The acute sera were taken not later than the fifth day after the onset of illness and the convalescent sera on the 1–20th (mean and median 15th). Group B: 14 subjects born in 1940–56,

TABLE 1 *HI Antibodies to Influenza A/Mayo Clinic/103/74 (HswIN1) in Sera Collected in Spring 1969 and Autumn 1975 from Subjects Born in 1892 1921*

Sera collected in:	Age of subjects (in 1975) Median	Mean	S.D	Frequency of seropositive* sera	Geometric mean titre of seropositive sera	
1969	64	64.5	8.4	22/24 91 7 per cent	87	P<0.05
1975	64	64.2	8.1	50/54 92.6 per cent	158	

Antibodies in titre of ≥12.

clearly after the era of the HswIN1 subtype. The acute sera were collected as in group A and the convalescent sera on the 11–22th day (mean and median 16th) All sera were stored at +4 C.

The first collection was studied for HI antibodies to A/Mayo Clinic/103/74 (HswIN1) and A/Port Chalmers/1/73 (H3N2) the second collection only for antibodies to A/Mayo Clinic/103/74 and the third collection for antibodies to the fiv influenza A strains listed in Table 2.

In HI tests, the technical performance principles presented by *Robinson & Dowdle* (17) were followed. Details of the procedure have been described previously (16) To remove nonspecific inhibitors, the sera were treated with cholera filtrate (Philips-Duphar B.V Holland)

RESULTS

Fig. 1 illustrates the occurrence of HI antibodies to influenza A/Mayo Clinic/103/74 (HswIN1) in sera collected in autumn 1975 There was a striking age correlation the frequencies of seropositive *subjects (antibodies* in a titre of ≥12) among those born before 1920, in 1920–30 and after 1930 were 91 per cent, 55 per cent and 2 per cent, correspondingly This pattern corresponds to a frequency of about 25 per cent in the Finnish population when the age distribution at the end of 1974 (3) is taken *into* account. Low titres from 12 to 24 were recorded only among subjects born in 1916–29 The anti-A/Port Chalmers/1/73 (H3N2) antibody curve exhibited quite a different pattern as compared with anti A/Mayo Clinic/103/74 antibodies. The average level of the former antibodies was about the *same in* each of the eight age groups of Fig 1 The same was also true of frequencies of seropositive subjects, which ranged from 50 to 63 per cent.

In the 1969 collection there was a lower level of HI antibodies to A/Mayo Clinic/103/74 than in sera collected in autumn 1975 and chosen to exhibit the same distribution by the year of birth (Table 1)

Results of homologous and heterologous antibody responses of influenza A patients from the H3N2 epidemic of 1975–76 are summarized in Table 2. Please note that 1) The acute phase specimens of the younger patients (born in 1940–56) possessed no anti body to A/PR/8/34 or A/swine-like viruses, nor was it possible to demonstrate a rise of these antibodies in any of the patients. 2) The acute phase specimens of the older patients (born in 1899–1918) rarely proved to be seronegative for antibodies to A/swine like viruses. About half of the patients showed a two-fold or higher rise in these antibodies. The highest rise was from a titre of 24 to 384

DISCUSSION

The presence of antibodies to influenza A/Mayo Clinic/103/74 (HswIN1) in the overwhelming majority of the subjects born before 1930 is in accordance with findings in other countries in spring 1976 (24) This age-related pattern suggests a high infection rate during the era of HswIN1 viruses. It is particularly interresting to note that in Finland the average population density during that era, e.g in 1926 was only 10.4 inhabitants/km² (20)

TABLE 2. *Response of HI Antibodies d ring the Epidemic of 1975–76 in 20 Influenz A Patients Born in 1899–1918 (Patient Group A) and in 14 Patients Born in 1940–58 (Patient Group B)*

Patient group	Strains used as antigens		Number (%) of patients with a titre of ≥12 in acute sera	HI antibodies: Geometric mean titre: *Acute sera*	*Conval. sera*	*X fold rise*	Number (%) of patients showing increase in titre: ≥2 fold	≥4-fold
A	A/Finland/23/75‡	(H3N2)	0 (0 %)	6	262	43 7x	20 (100 %)	20 (100 %)
	A/Hong Kong/8/68	(H3N2)	10 (50 %)	12	440	36 7x	20 (100 %)	20 (100 %)
	A/PR/8/34	(H0N1)	14 (70 %)	15	22	1.5x	7 (35 %)	1 (5 %)
	A/swine/1976/31	(Hsw1N1)	19 (95 %)	81	146	1.8x	10 (50 %)	4 (20 %)
	A/X 53†	(Hsw1N1)	17 (85 %)	50	81	1.6x	9 (45 %)	3 (15 %)
B	A/Finland/23/75	(H3N2)	0 (0 %)	6	101	16.8x	14 (100 %)	14 (100 %)
	A/Hong Kong/8/68	(H3N2)	12 (86 %)	50	403	8.1x	14 (100 %)	12 (86 %)
	A/PR/8/34	(H0N1)	0 (0 %)	6	6	1.0x	0 (0 %)	0 (0 %)
	A/swine/1976/31	(Hsw1N1)	0 (0 %)	6	6	1.0x	0 (0 %)	0 (0 %)
	A/X 53	(Hsw1N1)	0 (0 %)	6	6	1.0x	0 (0 %)	0 (0 %)

* Individual titres of <12 are regarded as 6.
‡ An epidemic strain in winter 1975–76 antigenically close to A/Victoria/3/75.
† A recombinant antigenically identical to A/Fort Dix/741/76.

There are some explanations for the accumulation of low titres (12–24) of anti A/Mayo Clinic/103/74 antibodies in the age group of subjects born in 1916–29 1) The subjects may possess a relatively small population of cross-reactive memory cells, e.g. as a result of less numerous infections with the A/swine-like viruses. 2) The virus strains originally responsible for the anti A/swine like antibodies of the younger subjects were not as close to A/Mayo Clinic/103/74 as the preceding strains of the older subjects.

Numerous studies have demonstrated a correlation between resistance against influenza and pre-epidemic level of serum HI antibody (16) The age-related distribution of antibodies to A/Mayo Clinic/103/74 suggests that an overwhelming majority of individuals over 50 years of age is not susceptible to an infection with A/swine like viruses. Thus they would not as a rule belong to a high-risk group if a new era of these viruses begins in the near future. In this connection one should also note that during the first epidemic of the H3N2 viruses in 1968–69 the oldest age groups were also partly protected (11 19) During the pandemic of 1918 there was a peak of morbidity and mortality at age 30 (4) and the frequency of influenza and pneumonia was comparatively low in older persons (7 12)

The comparison of sera collected in spring 1969 and autumn 1975 suggests that the level of antibodies to A/swine-like viruses, in the subpopulation of human beings born before 1920, has not decreased during the era of H3N2 viruses. In the 1975 collection the antibody level was observed to be even higher but the comparison may not be quite fair as the 1969 sera had been stored in less than ideal conditions.

A low but clear response of antibodies to A/swine-like viruses was frequently recorded during the H3N2 epidemic of 1975–76 in the influenza A patients born before 1918. Thus it seems obvious that cross-stimulation caused by the recent H3N2 viruses is one determinant responsible for the current high level of antibodies to A/swine-like viruses. Since their appearance in 1968, viruses of the H3N2 subtype have caused epidemics more often than their predecessors, the H2N2 viruses. With the exception of one winter (1970–71) outbreaks have occurred every year in Finland since the epidemic of 1971–72 the infection frequency in follow up samples has varied from 17 to 28 per cent (1 16)

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ACQUIRED RESISTANCE AGAINST *LISTERIA MONOCYTOGENES* IN RED MICE AND CF1 MICE IMMUNIZED WITH STRAINS OF BCG OR *MYCOBACTERIUM TUBERCULOSIS*

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Jespersen, A. Acquired resistance against *Listeria monocytogenes* in red mice and CF1 mice immunized with strains of BCG or *Mycobacterium tuberculosis*. Acta path. microbiol. scand. Sect. B, 84 379–385 1976.

Groups of red mice and CF1 mice immunized intravenously with varying doses of a weak or a strong strain of BCG or a strain of *Mycobacterium tuberculosis* were challenged 5 weeks after immunization with 0.1 or 0.2 ml 10^{-3} *Listeria monocytogenes* injected intravenously simultaneously with a non-immunized control group. The acquired resistance was determined on the basis of the number of survivors and the survival times of the animals that died spontaneously. In the red mice, the strong BCG strain induced a definitely higher resistance than the weak strain, and the *M. tuberculosis* strain a slightly higher resistance than the BCG strains. As in red mice, the resistance of CF1 mice was higher in animals immunized with *M. tuberculosis* than in those immunized with the BCG strains. However, the difference in the survival times of mice immunized with the two strains of BCG was much less than in red mice, and was only clearly significant as regards one of the doses used. The relationship between the virulence of a mycobacterial strain and its ability to induce acquired resistance against an infection with listeria or against an infection with virulent tubercle bacilli is discussed. It is concluded that red mice are more suitable than CF1 mice for evaluation of the protective potency of a BCG strain.

Key words: *Listeria monocytogenes*; red mice; CF1 mice; BCG-induced resistance; *M. tuberculosis*-induced resistance; potency of BCG strains.

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A previous study in red mice (*Jespersen* 1976 a) has shown that BCG injected intravenously induced a resistance to a listeria infection which could be demonstrated on the basis of the survival times after challenge 5 weeks following immunization. The degree of the resistance induced was significantly higher in animals immunized with a strain of BCG which was strongly virulent for hamsters, than in those immunized with a weakly virulent strain.

In order to elucidate the relationship between the virulence of a strain and its ability to protect against infection with listeria, the animals in the present study were immunized with the above mentioned strains of BCG and with a virulent strain of *M. tuberculosis*

TABLE 1 *Survival Times in Days of Red Mice and CF1 Mice Immunized Intravenously with Varying Doses (Viable Units) of Strains of BCG Prague BCG Copenhagen and M.* tuberculosis, *and Challenged Together with a Non Immunized Control Group 3 Weeks Later with 0 1 ml 10^{-8}* Listeria monocytogenes *Injected Intravenously*

Non-immunized controls	BCG Prague 3.8×10^4	BCG Prague 3.8×10^5	BCG Prague 3.8×10^6	BCG Copenhagen 6.5×10^4	BCG Copenhagen 6.5×10^5	BCG Copenhagen 6.5×10^6	*M tuberculosis* 7.0×10^5	*M tuberculosis* 7.0×10^6	*M tuberculosis* 7.0×10^7
Red mice									
3	3	2	3	3	4	5	3	s	6
3	3	3	3	3	5	s	7	s	s
4	3	3	3	4	5	s	s	s	s
4	3	3	3	4	5	s	s	s	s
5	3	4	3	4	s	s		s	s
5	3	4	3	5	s	s	s	s	s
s	3	4	4	5	s	s	s	s	
s	4	4	4	6	s	s	s	s	
s	4	s	4	7		s	s	s	s
s	4	s	5		s	s	§	s	§
CF1 mice									
3	3	2	4	3	3	5	10	s	10
3	3	3	5	3	4	s	s	s	s
3	3	3	s	3	4	s	s		s
3	3	4	s	4	5	s	s	s	
3	3	4	s	4	5	s	s	s	s
3	3	s		4	8		s	s	s
3	3			4	s	s	s	s	s
3	3		s	4	s	s	s	s	s
4	3	s	s	4		s	s	s	s
9	s	s	§		s	s	s	s	s

s = Survivor
§ = Died in the interval between immunization and challenge
Underlined figures = median survival time.

In addition to red mice, CF1 mice were used in order to examine which of the species is most suitable for demonstration of differences between strains of BCG

MATERIAL AND METHODS

The study was performed simultaneously with a previously published work on tuberculin shock in red mice and CF1 mice immunized with various strains of mycobacteria (*Jespersen* 1976b) For details of the mice and their distribution in the experimental groups, reference should be made to that paper The same applies to the origin of the mycobacterial strains, and the preparation of the suspensions for immunization of the mice for which cultures grown in Dubos fluid Tween medium were used. The result of a virulence test of the BCG strains in hamsters is also reported in that work.

The mycobacterial strains used were the BCG strains Prague and Copenhagen, the former of which was weakly virulent for hamsters and the latter considerably stronger and also a strain of *M tuberculosis* which was highly virulent for guinea pigs

Experimental

Expt. I Groups of 10 red mice and 10 CF1 mice were immunized intravenously in a tail vein with 0 1 ml of an undiluted culture, 0.1 ml of a tenfold diluted culture, and 0 1 ml of a hundredfold diluted culture of the three strains. The number of viable units in the smallest immunization dose, determined by inoculation on Löwenstein Jensen medium of suitable dilutions, was 3.8×10^4 for BCG Prague, 6.5×10^4 for BCG Copenhagen, and 7.0×10^5 for *M tuberculosis* The reason for the latter figure being so high is that this suspension was treated with ultrasonics. Three weeks

after immunization, the mice together with a non-immunized control group were challenged intravenously with 0.1 ml of a standard suspension of *Listeria monocytogenes* diluted a hundred times. This dose corresponds to about 2×10^4 viable organisms.

Expt 2 Groups of 10 red mice and 10 CF1 mice were immunized intravenously on the same day and with the same suspensions of the three mycobacterial strains as in Expt. 1 but with doses which were twice as large. The dose used for challenge 3 weeks later was also twice as large.

In both experiments the mice were immunized and challenged in random order. The survival times after challenge were used for evaluation of the acquired resistance. The mice were observed for 10 days after challenge.

Statistical analysis. The comparisons between various groups of animals were carried out by Wilcoxon rank-sum test (1945) in a modification compiled by Olsen Larsen (to be published). The survival times were divided into four groups, i.e. 2–3, 4, 5–10 and > 10 days. P values < 5 per cent are considered significant and are shown in text in brackets.

RESULTS

Expt 1

Table 1 shows the survival times in days of red mice and CF1 mice immunized intravenously with varying doses of BCG Prague, BCG Copenhagen and *M tuberculosis* and challenged 3 weeks later intravenously together with a non-immunized control group with 0.1 ml 10^{-2} *Listeria monocytogenes*.

Red Mice

The median survival time, determined on the basis of the survival times of the two middle animals *in the group* (underlined in table) was 5 days for the non-immunized control animals. Four mice survived the infection.

No increase in resistance could be demonstrated in the animals immunized with BCG Prague. All the mice died, except two immunized with the medium dose. The median survival times for the three groups were 3, 4 and 3 days.

The mice immunized with BCG Copenhagen and *M tuberculosis* developed a considerable degree of resistance. In the groups given BCG Copenhagen, the survival times and the number of survivors increased the higher the dose, while no dose-dependence could be demonstrated for the groups immunized with *M tuberculosis*. The difference in the acquired resistance was marked between the groups immunized with the BCG strains, but was less pronounced between those immunized with BCG Copenhagen and *M tuberculosis*. Since the virulence of *M tuberculosis* is higher than that of BCG for red mice, it would be presumed that considerably smaller doses than those used here would induce maximum resistance. It is therefore justifiable to compare the group immunized with the smallest dose of *M tuberculosis* with those given BCG Copenhagen. There were seven survivors in the first named group and 1, 6 and 9 in the latter group according to increasing dose.

Statistical analysis. The survival times in the groups immunized with BCG Prague are not mutually different. The survival times of the group immunized with the medium dose are not significantly different from those of the non-immunized control group. However the survival times for the groups immunized with the largest and smallest doses are significantly lower than those of the control animals. The explanation for this is presumably that the challenge dose is within a range where even quite small differences in the number of viable units in the various doses have a considerable effect on the survival times. The difference in the survival times for the groups immunized with BCG Prague and BCG Copenhagen is significant for all doses (p = 0.6 per cent, 0.4 per cent and 0.01 per cent).

The group immunized with the lowest dose of *M tuberculosis* is significantly different from the corresponding group given BCG Copenhagen (p = 1.4 per cent) but not different from those immunized with the medium and largest doses.

CF1 Mice

The strain of *M tuberculosis* induced a considerable degree of resistance. In the groups immunized with the two smallest doses

TABLE 2. *Survival Times in Days of Red Mice and CF1 Mice Immunized Intravenously with Varying Doses (Viable Units) of Strains of BCG Prague, BCG Copenhagen and M tuberculosis, and Challenged Together with a Non Immunized Control Group 3 Weeks Later with 0.2 ml 10^{-4}* Listeria monocytogenes *Injected Intravenously*

Non-immunized controls	BCG Prague			BCG Copenhagen			*M tuberculosis*		
	7.6×10^4	7.6×10^5	7.6×10^6	13×10^4	13×10^5	13×10^6	14×10	14×10^6	14×10^7
Red mice									
2	3	3	3	5	7	4	4	6	s
2	3	4	3	5	s	5	5	9	s
3	3	4	3		s	9	s	s	s
3	3	4	4	s	s	s	s	s	s
3	3	4	4	s	s	s	s	s	s
3	3	4	4	s	s	s	s	s	s
3	3	4	4	s		s	s	s	
3	3	6	5	s	s	s	s	s	s
3	3	9	5	s	s	s	s	s	s
4	4	s	7	s	s	‡		s	s
CF1 mice									
2	2	2	5	2	1	4	5	9	7
2	3	2	s	2	3	7	7	s	7
2	3	3	s	3	4	s	s	s	8
3	3	3	s	3	4	s	s	s	9
3	3	3	s	3	5	s	s	s	s
3	3	3	s	3	7	s	s	s	s
3	3	3	s	3		s	s	s	s
3	4	4	s	4	s	s	s	s	s
3	5	4	s	4	s	s	s	s	s
4	8	5	s	4		s	s	s	s

s = Survivor
‡ = Died in the interval between immunization and challenge.
Underlined figures = median survival time.

Of the BCG strains, the resistance was lower than in the corresponding groups given *M tuberculosis* but there was no definite difference in the effect of the two BCG strains.

Statistical analysis The difference between the survival times in the groups immunized with the lowest dose of BCG Prague and BCG Copenhagen is significant (p = 1.5 per cent) but between the groups given the two largest doses the difference is not significant. The group immunized with the smallest dose of *M tuberculosis* is significantly different from those given the two smallest doses of BCG Copenhagen (p = 0.01 per cent and 1.7 per cent) but not from the group immunized with the largest dose

Expt 2

Table 2 shows the survival times in days of red mice and CF1 mice immunized intravenously with the twice as large doses as in Expt. 1 of BCG Prague, BCG Copenhagen and *M tuberculosis* and challenged 3 weeks later simultaneously with a non-immunized control group with 0.2 ml 10^{-4} *Listeria monocytogenes* (twice as high a dose as in Expt. 1)

Red Mice

In consequence of the higher challenge dose, all the animals in the non immunized control group died and the median survival time was 3 days.

The mutual difference in the effect of the BCG strains was more evident in this experiment than in the first. In the animals immunized with BCG Prague, only a small increase in resistance could be demonstrated. Only one animal survived the infection and the median survival times according to increasing doses were 3 4 and 4 days, as against 3 days in the non-immunized control group. In contrast, there was a considerable increase in the resistance of the animals immunized with BCG Copenhagen and *M tuberculosis* The median survival times were >10 days in all groups, and the number of survivors was 8, 9 and 6 in the BCG groups and 8, 8 and 10 in the *M tuberculosis* groups with increasing doses.

Statistical analysis. The survival times for the groups immunized with the two largest doses of BCG Prague are significantly prolonged in comparison with those of the control group ($p = 0.08$ per cent and 1.2 per cent) There is no effect with the smallest dose of BCG Prague. The survival times of the groups immunized with all doses of BCG Copenhagen and *M tuberculosis* are not mutually different, but all are significantly different from the groups immunized with BCG Prague ($p<0.01$ per cent in all cases)

CF1 Mice

Increased resistance was found in the groups immunized with the largest dose of BCG Prague, the two largest doses of BCG Copenhagen, and all doses of *M tuberculosis*

Statistical analysis The difference between the survival times for groups immunized with BCG Copenhagen and BCG Prague is not significant for the groups immunized with the smallest and largest doses. The difference is significant for the groups immunized with the medium dose ($p = 0.6$ per cent) which may be due to difference in dosage

There is a significant difference in the survival times in the groups immunized with the smallest dose of *M tuberculosis* and those given the two smallest doses of BCG (for both Prague groups $p<0.01$ per cent, and for the Copenhagen groups $p<0.01$ per cent and 2.6 per cent) whereas the difference between the former group and that immunized with the largest dose of BCG Copenhagen is not significant.

DISCUSSION

The acquired resistance to virulent tubercle bacilli which develops after vaccination with BCG or other mycobacterial strains has often been evaluated using white mice or other members of the murid family However these animals are far from being ideal experimental animals for this purpose. The resistance which develops is weak, and it has been possible for only a few workers to demonstrate systematically any differences between the immunogenic potency of viable and killed mycobacteria or of viable BCG vaccines prepared from various strains. This is irrespective of whether evaluation of the potency has been based on quantitative culture from organs at various intervals after challenge with virulent mycobacteria, or on survival times of the infected animals. *Prichová* (1975) who for many years has compared the immunogenic potency of different BCG strains, has reported that she abandoned the use of white mice for such experiments and now uses guinea pigs exclusively

Red mice are able to develop a considerable degree of acquired resistance to infection with virulent tubercle bacilli. By means of the survival method it is possible not only to evaluate the protective potency of weakly virulent or strongly virulent strains of BCG but also of strains of intermediate virulence (*Jespersen & Bentzon* 1964 a b) The fact that a greater degree of resistance develops in red mice than in white mice can be illustrated by two studies in which the experimental conditions were almost identical. *Siebenmann & Barbara* (1974) compared the protective effect of a strongly potent BCG strain (Connaught 146) and of a BCG strain of medium potency (Japan) on albino mice (Connaught breed) after intravenous challenge with a large dose of *M bovis* Th

median survival time for the non-vaccinated animals was 30 days, for those vaccinated with a large dose of the Japanese strain the median survival time was not prolonged significantly and for those vaccinated with the Connaught strain the median survival time was 54 days ($p < 0.01$ per cent) In a protective study in red mice vaccinated with BCG strains corresponding in potency to those used by the Canadian workers, and also challenged with a large dose of *M bovis* (*Jespersen & Bentzon* 1964 b) the median survival time was 20 days for the non-vaccinated animals, 80 days for those given a large dose of the strongly potent BCG Dubos Copenhagen, and 65 days for those vaccinated with the intermediate BCG Gothenburg

The present study has shown that after immunization with the strongest BCG strain, the degree of resistance developed to listeria infection is less in CF1 mice than in red mice, and that using the survival method CF1 mice are not suitable for evaluation of differences between the immunogenic capacity of BCG strains.

Neither Hall mice nor C3H mice seem to develop high degrees of resistance. *Ackerman* (1964) found that the resistance of Hall mice was greater after intravenous than after intraperitoneal immunization with *Listeria monocytogenes* This could be demonstrated by the culture method only and not by LD_{50} determination. In experiments aimed at establishing a model for the comparison of BCG and other immunizing agents by the culture method on C3H mice, *Bennedsen* had to use two doses of BCG (the second given 5 days before challenge) to obtain a sufficient degree of resistance (*Bennedsen & Olesen Larsen* 1975)

Protective studies against infection with virulent tubercle bacilli in red mice and guinea pigs immunized with BCG strains of varying virulence (*Jespersen & Bentzon* 1964 a, b 1967) showed that the acquired resistance is greater the more virulent the BCG strain. This applies also to resistance to listeria infection, as demonstrated in previous experiments on red mice with BCG strains Prague and Copenhagen (*Jespersen* 1976 a) and in the present study with the same BCG strains and a strain of *M tuberculosis*

Thus, the degree of acquired resistance which a mycobacterial strain is able to induce in a given animal organism, both against infection with virulent tubercle bacilli and against infection with listeria, is dependent primarily—and perhaps exclusively—on the ability of the strain to multiply in the organism in question.

It can be concluded from the present and previous studies that there is a positive correlation between the capacity of a BCG strain to protect against listeria infection, against *M bovis* infection in red mice (*Jespersen & Bentzon* 1964 a, b) and guinea pigs (*Jespersen & Bentzon* 1967) and consequently also against tuberculous infection in human beings (*Jespersen* 1971)

The writer is grateful to *Michael Weis Bentzon* Chief of the Biostatistical Department, Statens Seruminstitut, who has performed and described the statistical analysis.

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CROSSED IMMUNOELECTROPHORETIC ANALYSIS OF *BORDETELLA PERTUSSIS* ANTIGENS AND OF CORRESPONDING ANTIBODIES IN HUMAN SERA

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Hertz, J. B., Holby, N., Andersen, V. & Bækgaard, P. Crossed immunoelectrophoretic analysis of *Bordetella pertussis* antigens and of corresponding antibodies in human sera. Acta path. microbiol. scand. Sect. B, 84 386–394 1976

Forty-four antigens were demonstrated in sonicated preparations of *Bordetella pertussis* (B.p.) using crossed immunoelectrophoresis against antiserum obtained from rabbits. No qualitative differences between the four strains of the Danish pertussis vaccine were found. In preparations of B.p. culture medium, purified with respect to Lymphocytosis Promoting Factor (LPF) activity, one antigen was possibly related to LPF. In human sera, antibodies against five of the B.p. antigens were demonstrated by means of crossed immunoelectrophoresis with intermediate gel. Antibody production was demonstrable in children during the pertussis vaccination period and was most marked after the second vaccination.

Key words: *Bordetella pertussis*; immunoelectrophoresis; serum antibodies; pertussis vaccination.

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Many studies have been concerned with the antigenic structure of *Bordetella pertussis* (B.p.) and several fractions with different biological and immunological activities have been isolated (20, 21, 23); however, quantitative immunoelectrophoretic analysis and identification of the antigenic components of B.p. has not been reported. The immune response following human infection and vaccination has been analysed by epidemiological and serological methods (2, 8, 21), but quantitative immunoelectrophoretic methods permitting detailed analysis of the humoral immune response have not been exploited.

Quantitative immunoelectrophoretic methods have to an increasing degree been applied in the study of microbial antigens and antimicrobial antibodies because these methods are sensitive and permit identification and quantitation of antigens and precipitating antibodies without purification of the components (6, 14, 15). In the present study immunoelectrophoretic methods have been applied to the study of B.p. antigens, using rabbit antisera, and to the evaluation of pre-

cipitating antibodies against B.p. in sera from healthy adults and from children suffering from pertussis or undergoing pertussis vaccination.

MATERIAL AND METHODS

Bordetella pertussis *Antigens (B.p. Ag)*

The four strains of B.p. constituting the Danish vaccine (Statens Seruminstitut, strain Number (No.) 3803, 3825 3845 3860) were cultured separately on modified Bordet-Gengou medium containing horse blood and charcoal at 35 C for 4 days the bacteria were scraped off the plates, washed 3 times, and disintegrated by sonication as described previously (15) After centrifugation (48,000 g, 60 min, 4 C) equal volumes of the supernatants were mixed and stored at –30 C. The colloid concentration, as measured by refractometry using human IgG as standard, was 5.6 g/l. Immunoelectrophoresis (5) according to *Grabar & Williams* (1953) of 10 µl B.p.-Ag against 200 µl B.p.-Ab showed that most of the antigens migrated towards the anode. However at least 5 antigens migrated cathodically by the method of crossed immunoelectrophoresis employed in this study antibodies against these antigens will not be revealed.

Fractions of B.p. with Lymphocytosis Promoting Factor (LPF) Activity

B.p. fractions with LPF activity purified from the culture medium of phase 1 bacteria, Tohama strain were kindly donated by *Y. Sato* (National Institute of Health, Tokyo Japan) The methods of purification and the biological activities of these fractions have been described (22, 23 24) The protein concentration of fraction Z-F was 1.5 g/l and of fraction SDGC-1 0.2 g/l.

Rabbit B.p. Antiser (B.p. Ab)

Three rabbits were immunized intradermally with B.p.-Ag in Freund' incomplete adjuvant following the immunization and bleeding schedule of *Harboe & Ingild* (1973) Each animal received 100 µl B.p.-Ag per injection. IgG and IgA were purified and concentrated (12) Bleeding No. 1 was used for pilot experiments. Immunoglobulins from bleedings 2–6 were pooled, since immunoelectrophoretic analysis revealed similar antibody patterns. This preparation contained 34.2 g protein/l as determined by refractometry and was used for the experiments in the present and accompanying papers (4 17)

Sonicated culture medium run against B.p.-Ag in crossed immunoelectrophoresis revealed 2 precipitates these were not related to the B.p.-Ag/B.p.-Ab pattern to be described and absorption of B.p.-Ab with the culture medium did not change the B.p. Ag/B.p.-Ab pattern.

Immunoelectrophoretic Methods

Immunoelectrophoretic analysis of B.p antigens was performed by crossed immunoelectrophoresis according to *Weeke* (1973) on 5 × 5 cm glass plates as described previously (14 15) Optimal resolution was obtained if 2 µl B.p.-Ag were employed in the first dimension electrophoresis and 20 µl B.p Ab/cm² in the second dimension gel. Coomassie Brilliant Blue was employed to stain the precipitates.

Human antibodies against B.p. were identified by means of crossed immunoelectrophoresis with human serum in an intermediate gel (40 µl/cm²) using the B.p Ag/B.p Ab precipitation pattern as a reference according to *Axelsen* (1973) as described previously (14)

Quantitation of antibodies in human sera was carried out by comparing the area included by each precipitate in the intermediate gel to the corresponding areas in five standard plates with different concentrations of rabbit B.p Ab in the intermediate gel. The titres of the human antibodies were expressed in arbitrary units in relation to the concentration of rabbit antibodies in the standard plates according to *Svendsen & Axelsen* (1972) as described previously (14)

0 µl/cm² < titre 1 ≤ 1 µl/cm² < titre ≤ 5 µl/cm² < titre 3 ≤ 10 µl/cm² < titre 4 ≤ 20 µl/cm < titre 5 ≤ 40 µl/cm² < titre 6 ≤ 80 µl/cm² < titre 7 Control plates with saline in the intermediate gel were run simultaneously The precipitin score (6) of each serum was calculated.

The four strains of B.p and the various antigen preparations were analysed and compared by means of

1) Crossed immunoelectrophoresis against B.p. Ab. All runs were repeated using different antigen/antibody ratios (15)

2) Tandem-crossed immunoelectrophoresis according to *Krøll* (1973 a) as described previously (15) with B.p. Ag against B.p.-Ab (or added to the first dimension well together with B.p.-Ag) The first dimension electrophoresis was run with antigen in the following concentrations 2 µl undiluted, 1 µl undiluted, and 1 µl diluted 1.5 with the barbital buffer used for the electrophoresis (15)

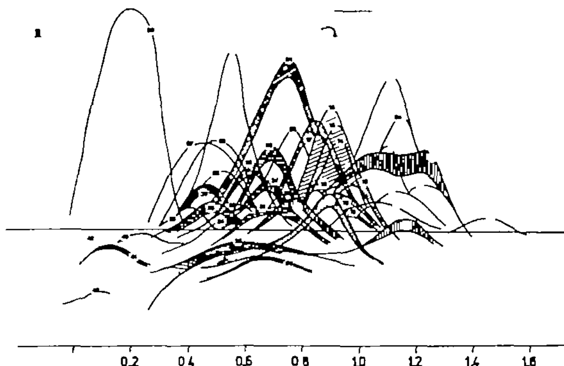
3) Crossed-line immunoelectrophoresis according to *Krøll* (1973 b) as described previously (15) with the antigen preparation in question in the intermediate gel (absorption of antibodies *in situ*) The intermediat gel contained 20 µl, 40 µl or 80 µl cm² of the antigen solution, and the reference gel contained 20 µl B.p. Ab/cm²

The sensitivity of th method as judged by

Fig 1 The B.p. Ag/B p -Ab reference precipitation pattern.

A Crossed immunoelectrophoresis of B.p.-Ag against B.p.-Ab. Saline in the intermediate gel. Anode to the right in the first dimension and at the top in the second dimension electrophoresis.

B Drawing of A and enumeration of the precipitates. An arbitrary scale of migration velocity relative to human serum albumin (1.0) is indicated below



model experiments was found to correspond to 1/64 μg human albumin applied in 1 μl samples.

Human Sera

Sera from 3 groups of persons were examined for precipitating antibodies against B p.

a) 16 healthy adults were tested once

b) 11 healthy children who at 5 weeks, 9 weeks, and 10 months of age were vaccinated against whooping cough. These children were studied immediately before and 6 days after each vaccination, but only three children completed the investigation. The mothers of the children were tested once in connection with the first vaccination.

c) Five children, of ages between 3 and 27 months, with whooping cough verified by culture were tested once after at least 3 weeks illness.

Gammaglobulin

Human gammaglobulin (10 per cent, Statens Seruminstitut, Denmark) The preparation contains mainly IgG.

RESULTS

The immunoelectrophoretic pattern of B p. sonicate (B p -Ag) using rabbit antisera (B.p.-Ab) is shown in Fig. 1 A B. Forty-four precipitation lines are observed. These are

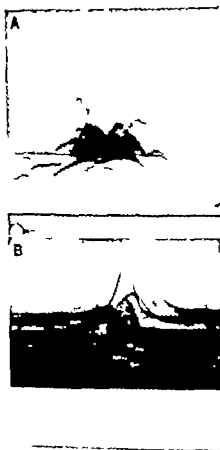


Fig 2 Comparison of B p. strain 3803 with B p. Ag.

A: Crossed immunoelectrophoresis of B.p strain 3803 against B.p.-Ab. Saline in the intermediate gel.

B: Crossed-line electrophoresis of B.p.-Ag against B.p.-Ab and B p. strain 3803 in the intermediate gel (absorption of antibodies *in situ*) All of the precipitates of the reference pattern have been removed or elevated and corresponding straight precipitation lines showing reaction of identity with the elevated reference precipitates are seen in the reference gel.

enumerated from the anode and the migration velocity of the various antigens is indicated in relation to human serum albumin (Fig 1 B) Pre-immunization sera from the three rabbits contained antibodies corresponding to precipitates No. 11 26, 35 and 40 with titres ranging from 1 to 5.

Crossed-line immunoelectrophoresis of the antigens from the 4 strains of B p. demonstrated that there were no qualitative differences (i.e. in number and identity of antigens) between the strains (Fig 2) To establish differences in the concentrations of the individual antigens will demand detailed planimetric investigations our preliminary results indicate that such quantitative differences between the strains do exist.

Fractions of B.p possessing LPF activity were studied in crossed-line and tandem-crossed immunoelectrophoresis. As seen in Fig. 3 fraction Z-F contained antigens cross-reacting immunologically with the antigens No. 26 and 40 besides, 2 or 3 faint non identifiable lines were seen. Fraction SDGC-1 contained one antigen which cross-reacted immunologically with the antigen No 40 (Fig. 3 E)

Examples of the antibody patterns in human sera and gammaglobulin are shown in Fig 4 The prevalence of precipitins in human sera is given in Table 1 and the precipitin score in Fig. 5 Antibodies against 4 of the 44 antigens demonstrable in the rabbit reference system were present. All persons tested had precipitin No. 26 approximately half of the persons had precipitin No. 11 All children had precipitins before the first pertussis vaccination In all cases these were identical to the precipitins of the mother and were found in the same or lower titre. It appears from Table 1 and Fig 5 that antibody production was demonstrable during *the course of vaccination* precipitins already present increased in titre and new precipitins appeared. This was particularly marked after the second vaccination.

The 5 children who were suffering from whooping cough had precipitins No 26 and 11 in their serum, but not precipitins No. 2 and 20 which developed in the vaccinated children (Table 1 and Fig 5)

Human gammaglobulin preparations (Fig 4) contained precipitins No 11 20 and 26 at high titres besides, precipitin No 7 was found low titre

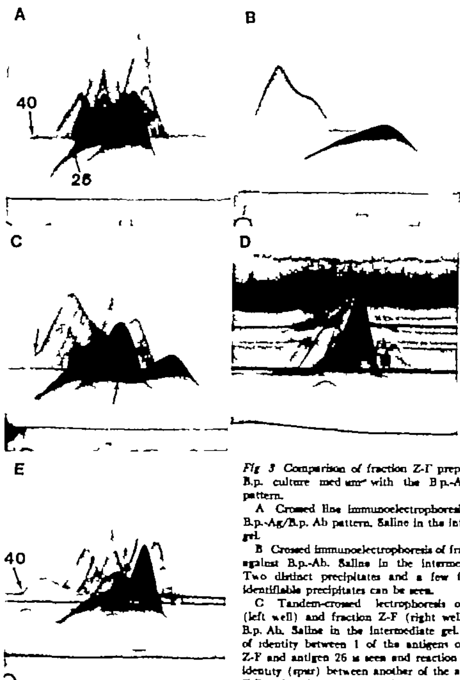


Fig 3 Comparison of fraction Z-F prepared from B.p. culture med um with the B p.-Ag/B.p.-Ab pattern.

A Crossed line immunoelectrophoresis showing B.p.-Ag/B.p. Ab pattern. Saline in the intermediate gel.

B Crossed immunoelectrophoresis of fraction Z F against B.p.-Ab. Saline in the intermediate gel. Two distinct precipitates and a few faint non-identifiable precipitates can be seen.

C Tandem-crossed lectrophoresis of B.p.-Ag (left well) and fraction Z-F (right well) against B.p. Ab. Saline in the intermediate gel. Reaction of identity between 1 of the antigens of fraction Z-F and antigen 26 is seen and reaction of partial identity (spur) between another of the antigens of Z-F and antigen 40 (arrows)

D Crossed-line immunoelectrophoresis of B.p Ag against B.p.-Ab, with fraction Z-F in the intermediate gel. Precipitates No. 26 and 40 (Fig. 1 B) have been removed from the reference pattern. Other precipitates (less distinct in B) have also been removed or elevated from the reference pattern. Straight precipitation lines showing reaction of identity with the elevated reference precipitates re seen. *If A D in Fig. 3 are compared, it can be concluded* that the Z-F antigens corresponding to the 2 distinct precipitates in B are immunological cross-reactive with antigen No. 26 and 40 of the B.p. Ag/B.p.-Ab pattern.

E: Crossed-line immunoelectrophoresis of B.p. Ag against B.p.-Ab with fraction SDGC-1 in the i ter mediate gel. A straight precipitation line is seen, precipitate No. 40 (Fig. 3 A) shows reaction of

identity (arrow) with this line and has been elevated from the reference pattern. None of the other precipitates of the B.p.-Ag/B.p Ab pattern have been elevated or removed, thus it can be concluded that the fraction SDGC-1 contains an antigen immunologically cross-reactive with antigen No. 40

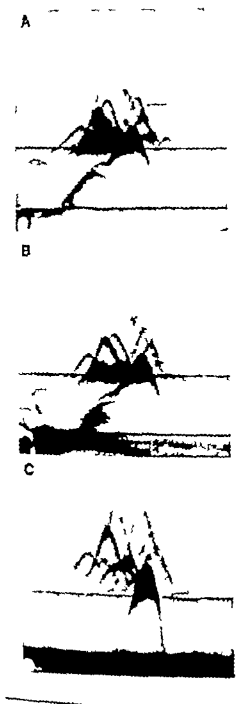


Fig. 4. Examples of crossed immunoelectrophoresis of B.p.-Ag against B.p.-Ab with intermediate gel containing human serum. Compare with Fig. 1 A-B.

A: Healthy adult. Precipitate No. 11 in titre 2 and No. 26 in titre 2.

B: Child 6 days after the third pertussis vaccination. Precipitate No. 2 in titre 2, No. 11 in titre 2 and No. 26 in titre 2.

C: Human gammaglobulin. Precipitate No. 7 in titre 1. No. 11 in titre 3. No. 20 in titre 2, and No. 26 in titre 3.

DISCUSSION

The present study shows that it is possible to employ the crossed immunoelectrophoretic methods in the study of *B. pertussis* components and of the immune response to these components. Employing sera from immunized rabbits, 44 antigens were demonstrated in B.p. sonicates. Further information might be obtained by 1) including the IgM class of rabbit antibodies which are lost during the purification procedure employed in this study (12), 2) increasing the number of rabbits used for production of B.p.-Ab (16) and 3) using detergent in the preparation of B.p. Ag (7).

No qualitative antigenic differences between the four strains used in the Danish pertussis vaccine were found, but preliminary studies indicate that differences in the concentrations of individual antigens exist. Other methods (3, 9, 21) have previously demonstrated differences between different *B. pertussis* strains, but their significance for vaccination and infection remains uncertain.

Two B.p. fractions were studied which were purified with respect to lymphocytosis promoting factor (LPF) activity. Few precipitates were found in these two fractions by immunoelectrophoretic analysis. Both contained antigens cross-reacting with antigen No. 40 which points towards the possibility that this antigen is related to LPF.

All examined human sera contained antibodies against B.p. components: antibodies against a total of 4 B.p. antigens were demonstrated. This is comparable in number to the four antibodies against B.p. which were found in sera from non-immunized rabbits.

TABLE 1 *Prevalences of the Different* B. pertussis *Precipitins Found in Sera from Normal Adults Children with Whooping Cough and Healthy Children During Pertussis Vaccination: Number of Sera Containing the Precipitin/Total Number of Sera in Each Group*

Precipitin number	Adults	Children with whooping cough	Children during vaccination					
			1 vaccination		2' vaccination		3 vaccination	
			B*	A	B	A	B	A
2	0/16	0/5	0/11	0/9	0/7	1/5	0/3	3/3
11	7/16	4/5	4/11	6/9	5/7	5/5	3/3	3/3
20	1/16	0/5	0/11	0/9	1/7	1/5	2/3	2/3
26	16/16	5/5	11/11	9/9	7/7	5/5	3/3	3/3

B = before, A = 6 days after vaccination.

The occurrence of these antibodies in non immunized rabbits seems less surprising considering that *B. pertussis* immunologically is extensively cross-reacting with *B bronchiseptica* which frequently is colonizing rabbits (17) Concentrated human gammaglobulin preparations showed the highest titres of precipitins, and one (No 7) was solely demonstrated in these concentrated preparations.

Precipitin No 11 which was found in ap-



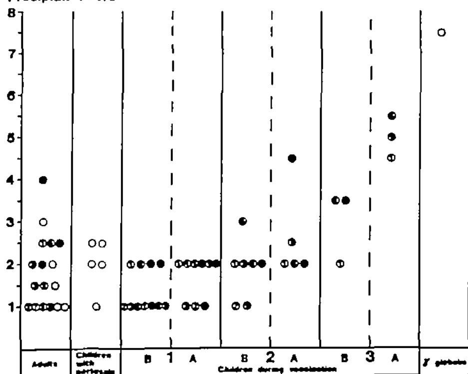


Fig 5 Precipitin scores of sera in the groups of human subjects studied. The score of each serum was calculated as the sum of the following 1 point for an antibody with titre 1 1½ points for titre 2, points for titre 3, etc. The numbers in the circles identify the individual vaccinated children and their mothers. B. = before A = after

proximately half of the human sera studied was found to cross-react with many other bacterial species (17). Another precipitin No. 26 was found in all human sera; the reactivity of this precipitin seems limited to Bordetella since the only cross-reaction found was with *B. bronchiseptica*. Both these precipitins increased in titre during vaccination (Table 1).

Infants studied before the first pertussis vaccination had the same antibodies as their mothers, in identical or lower titre; this is compatible with transplacental transport. Children with pertussis showed the same pattern as normal adults, thus the number of antibodies was not increased (Table 1). In the small material of vaccinated children, an increase in number and titre of precipitins was demonstrated, in particular after the second and third vaccination. The highest precipitin score in all human groups examined was found at the end of the vaccination period (Fig. 5).

The importance of local mucosal immunity has been established in several infections (27), but in B.p. infection and vaccination the immunological mechanisms are not fully comprehended (10, 13, 21). Quantitative immunoelectrophoretic methods have been applied to the study of antibodies in bronchial secretions (25) and may also prove useful for investigations on respiratory tract antibodies against B.p.

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CROSS-REACTIONS BETWEEN *BORDETELLA PERTUSSIS* AND TWENTY-EIGHT OTHER BACTERIAL SPECIES

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Høiby N Hertz, J B. & Andersen, V Cross-reactions between *Bordetella pertussis* and twenty-eight other bacterial species. Acta path. microbiol. scand. Sect. B, 84 395–400 1976.

Cross-reactions between *B. pertussis* and 28 other bacterial species were studied by various quantitative immunoelectrophoretic methods. A sonicated *B. pertussis* antigen preparation and a corresponding pooled rabbit antiserum were used as reference system. Two of the *B. pertussis* antigens were cross-reactive with antigens from 17 respectively 19 other bacterial species, mainly gram-negative species. As judged by absorption of antibodies, the degree of cross-reactivity of these *B. pertussis* antigens with antigens from other species was found to be in the range 25–50 per cent. Antigens from *B parapertussis* and *B. bronchiseptica* were found to cross-react very extensively with *B. pertussis* and only 4 respectively 2, of the 44 antigens of the *B. pertussis* reference system could not be absorbed with antigens from these two *Bordetella* species.

Key words *Bordetella pertussis* cross-reactivity quantitative immunoelectrophoresis.

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The antigenic complexity of *Bordetella pertussis* as revealed by various quantitative immunoelectrophoretic methods (5) raises the question whether all the 44 antigens demonstrated are specific. Earlier investigations have described a close antigenic relationship between *B. pertussis* and other Bordetella species (1 3) recently *Høiby* (8) demonstrated the presence of 10 antigens in *P aeruginosa* which were cross-reactive with a number of other bacterial species and 2 of these antigens cross-reacted also with *B. pertussis*

The aim of the present investigation was to compare antigens from a broad spectrum of other bacterial species with antigens from *B pertussis* A *B. pertussis* antigen (B.p Ag) antibody (B.p.-Ab) system was used as reference (5) and various quantitative immunoelectrophoretic methods were employed to compare antigens from other bacterial species with the B.p.-Ag/B.p. Ab reference system.

MATERIALS AND METHODS

The Bordetella pertussis *Antigen Antibody Reference System* (B p. Ag/B.p Ab) is described in detail in the preceding article (5) where also the 44 precipitates are enumerated.

Preparation of Antigens from 28 other Bacterial Species

The origin of most of the bacteria recorded in Table 1 and the preparation of antigens have been described previously (8) *Listeria monocytogenes* was isolated in this laboratory from a case of meningitis. The 4 *P aeruginosa* strains have been described (6) *Brucella abortus Bang*, *B. bronchiseptic* and *B. parapertussis* were cultured on solid media and suspended in saline. The antigens were obtained by sonication, as previously described (8) The colloid concentrations of these antigens were 3 2–18.9 g/l (refractometry using human IgG as standard)

Immunoelectrophoretic Methods

Antigens from each of the 28 bacterial species were compared with the B.p. Ag by means of a series of quantitative immunoelectrophoreses. Each antigen preparation was run 1) in crossed immunoelectrophoresis according to *Weeke* (1973) as described previously (7) against B.p.-Ab 2) in tandem-crossed immunoelectrophoresis according to *Krøll* (1973 a) as described previously (8, 10) with B p.-Ag (or added to the same first dimension well as B.p.-Ag) against B p.-Ab 3) in crossed-line immunoelectrophoresis according to *Krøll* (1973 b) as described previously (8 10) with the antigen in question included in an intermediate gel between first and second dimension electrophoresis of B.p.-Ag against B p. Ab (absorption of antibodies *in situ*) The analytical series of electrophoreses were repeated at least twice using different antigen/antibody ratios (8) The percentage of antibodies absorbed *in situ* in the crossed-line immunoelectrophoreses were estimated by comparison with sets of four standard plates containing 100 per cent, 75 per cent, 50 per cent, and 25 per cent, respectively of the original concentration of B.p.-Ab, as described previously (8, 10) The increase in enclosed area by given precipitate after absorption in situ can then be expressed as 100% 100%>–>75% 75%>–>50% 50%>–25% absorption of antibodies against the antigen in question absorption of less than 25 per cent was considered insignificant, bearing in mind the analytical variation of the present method (8, 10) The first dimension electrophoresis of the antigens was run with µl, 1 µl, or 1 µl diluted 1 5 with the barbital buffer used for electrophoreses (7) and appropriate combinations of these volumes in the same well or in the wells of the tandem-crossed immunoelectrophoresis. The intermediate gel of crossed-line immunoelectrophoresis contained 20 µl, 40 µl, or 80 µl/cm² of the antigen in question, or saline as control, and the reference gel contained B.p.-Ab 20 µl/cm² Immunoplates which were compared by these methods were always run simultaneously All the antigens of B.p.-Ag were stable at the mixing temperature of the agarose (40° C)

RESULTS

The results of the comparison of antigens from 28 different bacterial species with the *Bordetella pertussis* B p Ag/B.p. Ab reference system are given in Table 1 Examples of a comparative series of immunoelectrophoreses are given in Fig 1 A, B, C, and D Two antigens, numbered 11 and 28 in the reference system, were found in a wide range of bacterial species, mostly among gram-negative species. Number 11 was found in 19 different species and number 28 in 17 diferent species. Antigen number 37 was found in 3 bacterial species.

B parapertussis and *B bronchiseptica* were found to cross-react very extensively with *B. pertussis*. This leaves only 1 of the antigens of the reference system (number 21) as a possible *B pertussis-specific* antigen. Additional experiments showed that B.p. Ag could absorb all antibodies in B p.-Ab which reacted with antigens from *B. parapertussis* and *B bronchiseptica*. Apart from the antigens of *B parapertussis* and *B bronchiseptica* (Fig 2 A, B, C, and D) and one of the antigens of *P multocida* the cross-reactions between *B. pertussis* and other bacterial species were of minor degree (25–50 per cent as judged from the absorption of antibodies in *in situ* experiments)

The results obtained in the present study together with results previously published (8) and unpublished experiments where the B.p Ag/B.p.-Ab and the *P aeruginosa* St Ag/St. Ab reference systems are compared showed that antigens numbers 11 and 28 in the *B. pertussis* reference system correspond to antigen number 10 and number 24 respectively in the *P aeruginosa* St.-Ag/St.-Ab reference system (8, 10)

DISCUSSION

The results show that 2 of the *B. pertussis* antigens, numbers 11 and 28, cross-react with

TABLE 1 *Cross-reactions between* Bordetella pertussis *and other Bacterial Species. The Numbers Signify the Cross-reactive Antigens in the Reference System. The Number of Strains Tested and the Group Type or Collection Numbers Are Given in Parentheses*

Species	Cross-reactive antigens and percentage of cross-reactivity			
	100%	<100%–≧75%	<75%–≧50%	<50%–≧25%
Staphylococcus aureus (4 from each of the 4 phage groups)				
Streptococcus pyogenes (1 Group A)				
Streptococcus faecalis (1)				
Diplococcus pneumoniae (1 type 23F)				11–28
Bacillus cereus var *mycoides* (1 ATCC 11778)				
Corynebacterium species (1)				
Clostridium welchii (1)				11–28
Neisseria meningitidis (1 Group A)				11–28
Haemophilus influenzae (1 non-capsulate)				11–24–28
Pasteurella multocida (1)	37			11–28
Escherichia coli (1 O21 H27)				11–28
Salmonella typhi (1)				11–28
Shigella sonnei (1)				11–28
Citrobacter intermedius (1)				11–28
Enterobacter cloacae (1)				11–28
Klebsiella pneumoniae (1 type 35)				11–28
Serratia marcescens (1)				11–28
Proteus mirabilis (1)				11–28
Yersinia enterocolitica (1 type 3)				11
Flavobacterium meningosepticum (1)				
Bacteroides fragilis ss. thetaiotaomicron (1 VP15)				11
Vibrio cholerae (1 classica Inaba)				
Listeria monocytogenes (1)				11–28
Pseudomonas aeruginosa (4 O groups 3 5 6, 11)				11–28
Pseudomonas maltophilia (1)				
Brucella abortus Bang (1 strain 50)				
Bordetella bronchiseptica (1 "Leo")	42 antigens 100% cross-reactive*			
Bordetella parapertussis (1 strain 5681/50-51)	40 antigens 100% cross-reactive‡			

* Two *B. pertussis* antigens (no. 21 & 25) were not cross-reactive.
‡ Four *B. pertussis* antigens (no. 7 21 26 & 56) were not cross-reactive

antigens from a wide range of bacterial species. These cross-reactive antigens have been described previously using a *P. aeruginosa* antigen antibody system as reference (8 9) The distribution of these two antigens in the various bacterial species, as revealed by the present reference system, is very close to the distribution to be found if the *P. aeruginosa* reference system is used (8) Minor differences between the results obtained in these two studies are in evidence. In the present study antigen number 28 was found only in one gram-positive bacterial species (*B. cereus*) whereas the distribution of the corresponding antigen of *P. aeruginosa* (number 24 in the *P. aeruginosa* reference system) was also found to extend to gram-positive cocci (8) The reason for the differences between these two results is [illegible]ncertain but differences in the rabbits used for production of antisera may be [illegible] (2 4 11)

The [illegible] between *B. bronchi*[illegible] and *B. pertussis* has [illegible] to earlier reports [illegible] antigens (1 3) The [illegible] that the relationship is

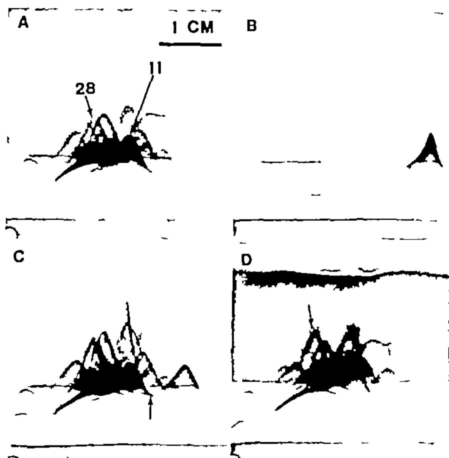


Fig 1 Comparison of antigens from *Salmonella typhi* with *Bordetella pertussis* antigens (B p.-Ag) using a pooled rabbit anti-*Bordetella pertussis* antiserum (B.p.-Ab)

A Crossed immunoelectrophoresis of B.p.-Ag against B.p. Ab. Saline in the intermediate gel. Forty-four precipitates were seen in this reference pattern. Precipitates No. 11 and 28 are indicated by arrows.

B Crossed immunoelectrophoresis of *Salmonella typhi* antigens against B.p.-Ab. Saline in the intermediate gel. Two distinct precipitates are visible.

C Crossed immunoelectrophoresis of B p Ag and *S typhi* antigens in the well (addition) against B p.-Ab. Saline in the intermediate gel A weak reaction of partial identity between one of the *S typhi* antigens (the anodic) and B.p. antigen No. 11 (arrow) is seen. Compare this plate with Fig. 1 A and B.

D Crossed-line immunoelectrophoresis of B p.-Ag against B.p.-Ab with *S typhi* antigens in the intermediate gel (absorption of antibodies *in situ*) Two of the precipitates of the reference pattern have increased in area (No 11 and 28 indicated by arrows) Compare this plate with Fig 1 A, B, and C (Technical First Dimension electrophoresis anode to the right. Second dimension electrophoresis anode at the top)

very close, as most of the *B pertussis* antigens of the reference system are 100 per cent cross-reactive with antigens from the two other species. In fact, only 1 of the antigens in the reference system was found to be *B pertussis* specific. Whether antigens which are specific for *B parapertussis* or *B bronchiseptica* actually exist as found in other studies by means of agglutination-absorption techniques (1 3) could not be confirmed in the present study Antisera raised against these two species would be necessary as the results of the present study indicate that the antigenic basis for differentiation between these 3 species is small

The nature of the cross-reactive antigens

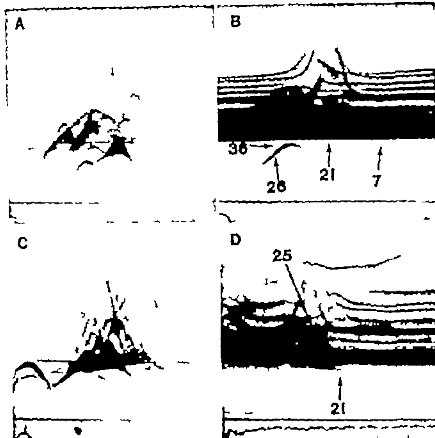


Fig. 2 Comparison of antigens from *Bordetella par pertussis* and *Bordetella bronchiseptica* with *Bordetella pertussis* antigens (B.p.-Ag) using a pooled rabbit anti-*Bordetella pertussis* antiserum (B.p.-Ab)

A: Crossed immunoelectrophoresis of *B. parapertussis* antigens against B.p.-Ab. Saline in the intermediate gel. Forty precipitates are visible.

B: Crossed-line immunoelectrophoresis of B.p.-Ag against B.p.-Ab with *B. parapertussis* antigens in the intermediate gel (absorption of antibodies *in situ*). Forty of the precipitates of the reference pattern have been removed or elevated, and corresponding straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel. Four of the reference precipitates have not been removed (indicated by arrows and numbers). The control reference-pattern of B.p.-Ag against B.p.-Ab can be seen on Fig. 1 A.

C: Crossed immunoelectrophoresis of *B. bronchiseptica* antigens against B.p.-Ab. Technique as that in Fig. 2 A. Forty-two precipitates are visible.

D: Crossed-line immunoelectrophoresis of B.p.-Ag against B.p.-Ab, with *B. bronchiseptica* antigens in the intermediate gel. Forty-two of the precipitates of the reference pattern have been removed or elevated, and corresponding straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel. Two of the reference precipitates have not been removed (indicated by arrows and numbers). Technique as that in Fig. 2 B. Compare with the control reference pattern of B.p.-Ag against B.p.-Ab in Fig. 1 A.

demonstrated in the present study remains so far obscure. It was shown that the 2 corresponding antigens of the *P. aeruginosa* system were not related to the Forssman antigen or to endotoxin (8) and according to experiments, not yet published, carried out in this laboratory they do not rea[ct] with the C-reac[tive] [pro]t[ei]n. The prese[n]c[e] [o]f other antigens wh[ic]h [c]ros[s-]react be[t]ween bacterial species is [we]ll know[n] [and] it will be interesting to

learn whether these antigens are related to the cross-reactive antigens demonstrated in the present investigation. The various quantitative immunoelectrophoretic methods should be well suited to investigate and quantitate such relationships.

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ULTRASTRUCTURAL STUDIES ON THE ENDOGENOUS DEVELOPMENT OF *EIMERIA BRUNETTI*

I Schizogony

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The schizonts of *Eimeria brunetti* were studied in the epithelial cells of the small intestine of infected chickens. The morphology of the host/parasite relationship was typical of that reported for other *Eimeria spp*. The initial dedifferentiation of the infecting organism generally occurred within the parasite cytoplasm but it was also observed that cytoplasm containing organelles could be budded off into the parasitophorous vacuole. Development into the schizont was accompanied by cytoplasmic growth and nuclear division. During nuclear division an eccentrically located nuclear spindle was present. Merozoite formation was initiated just below the limiting membrane of the schizont and was associated with the final nuclear division. The merozoites developed as protrusions from the schizont surface and merozoite organelles developed within these cytoplasmic projections. From this early stage the developing merozoite grew and matured and the fully formed merozoites were found attached to a small residual mass of schizont cytoplasm. The 1st generation schizont is the only one which can be separately characterized. It differs from those of subsequent generations in a) possessing a refractile body b) being larger and producing a larger number of merozoites, c) possessing invaginations of the limiting membrane and d) the intra vacuolar folds of its parasitophorous vacuole are more extensive.

Key words *Eimeria brunetti*, chickens, ultrastructure, schizogony

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Eimeria brunetti is a species of coccidia infecting chickens. It is highly pathogenic for chickens (*Hein* 1974) and field outbreaks of disease caused by this parasite have been reported in the USA and Britain by *Levine* (1942) and *Davies* (1963) respectively

In contrast to the other 4 highly pathogenic species affecting chickens (*Eimeria tenella*, *E. necatrix*, *E. acervulina* and *E. maxima*)

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nothing is known of the ultrastructural aspects of the life cycle of *E. brunetti*.

In this report, the ultrastructure of the various stages in the process of asexual multiplication (schizogony) of *E. brunetti* occurring in the epithelial cells of the small intestine of the infected chickens, will be described and compared to the developmental stages described for the schizonts of other coccidia.

MATERIALS AND METHODS

Chickens: White Plymouth Rock chickens were reared and kept in a coccidia free environment using sterilised food and water.

Oocysts: A pure sample of sporulated oocysts of *Eimeri brunetti* was obtained from the Central Veterinary Laboratory, Weybridge, England. For details of the maintenance of this strain see *Hein* (1974).

TABLE 1. *The Variation of Infective Dose to Time of Autopsy*

Chicken No.	No. of oocysts fed	Autopsy/hours post infection
1	1,000,000	48
2	1,000,000	60
3	500,000	72
4	100,000	84
5	50,000	96
6	10,000	108
7	1,000	120
8	500	144

Six week old chickens, after faecal examination for coccidian oocysts, were tube fed a specific number of sporulated oocysts, which was calculated using a Fuchs-Rosenthal haemocytometer. The inoculum size varied depending on the time of autopsy (Table 1). The inoculum size was large in the chickens killed at short post infection (p.i.) periods to provide a large number of parasites for examination but at the longer p.i. periods the inoculum was progressively reduced to prevent excess host cell destruction during the initial development cycles. The first chicken was killed at 48 hours p.i., and the others at intervals of 12 hours, until 120 hours with the last chicken being killed at 144 hours p.i. Our results are based on observations made during 3 experiments of this type.

At autopsy the small intestine was removed and divided into eight portions. A part of each portion was used to make smears which were examined by light microscopy to determine the regions of highest parasite density. Samples from the portions containing the largest number of parasites were cut into 1 mm^3 cubes and placed in 6 per cent glutaraldehyde in cacodylate buffer, pH 7.2, and fixed at room temperature for a minimum of 4 hours. The tissue blocks were washed with 0.2 M sucrose in cacodylate buffer, post fixed in 1 per cent osmium tetroxide in veronal acetate buffer, pH 7.3, for 1 hour, followed by treatment *en bloc* with 2 per cent uranyl acetate in veronal acetate buffer pH 7.3 for 1 hour. Dehydration was performed through an ethanol series followed by a propylene oxide treatment after which the tissue was embedded in Vestopal-W. Sections were cut on a L.K.B. microtome, stained with magnesium uranyl acetate (*Frasca & Parks* 1965) and lead citrate (*Reynolds* 1963), and examined with a Philips E.M. 200 or E.M. 300 electron microscope.

The results reported in this paper are based on 250–300 electron micrographs.

RESULTS

The gross morphological changes accompanying the various stages of schizogony of *E. brunetti* are known from light microscopic examination (*Boles & Becker* 1954). Based on these earlier observations it was possible to select different stages of schizogonic development for a comparative study of their ultrastructural morphology.

The infecting organism (sporozoite or merozoite) was found in the epithelial cell close to the basement membrane lying within a parasitophorous vacuole (Fig. 1). This vacuole, probably formed by the host cell as a result of the parasite intrusion, was limited by a unit membrane with a number of evaginations in the form of intra vacuolar folds which appeared as two closely applied unit membranes (Fig. 3). It was impossible to determine whether all these folds remained attached to the membrane of the parasitophorous vacuole. In the epithelial cell, the established organism developed into the trophozoite which increased in size. As this conversion occurred there was a loss of the anterior organelles of the organism (i.e. conoid, rhoptries and micronemes) and of the sub-pellicular microtubules and the inner



Fig 1 A newly entered sporozoite possessing a nucleus, refractile body micronemes, and rhoptries lying within a parasitophorous vacuole. × 12,000

Fig 2 Part of a developing trophozoite from which a portion of cytoplasm seems to be extruded into the parasitophorous vacuole. The extruded cytoplasm contains some micronemes, and subpellicular microtubules, and it is limited by a two layered pellicle. In contrast, the trophozoite is limited by a single unit membrane × 45,000

Fig 3 Part of the limiting membrane of a parasitophorous vacuole showing intra-vacuolar folds. × 50 000

Figures 1–19 are all micrographs obtained from sections of epithelial cells of the small intestine of chickens infected with *Eimeria brunetti* and illustrate the asexual development of the parasite.

A double bar (=) on a micrograph represents 1 μm and a single bar (—) represents 100 nm.

The following abbreviations are used throughout C, Conoid ER Rough Endoplasmic Reticulum G, Golgi body IF Intra-vacuolar Folds LM, Limiting Membrane of the Parasite M, Microneme MI Mitochondrion MT Microtubule MV Multi-membraneous Vacuoles N Nucleus NP Nuclear Pole NU Nucleolus P Pellicle; PG Polysaccharide Granule PV Parasitophorous Vacuole R, Rhoptry RB, Refractile Body; RM, Residual Mass of Cytoplasm RP Rhoptry Precursor

layer of the pellicle This dedifferentiation generally occurred within the cytoplasm of the trophozoite but sometimes portions of cytoplasm containing these organelles were budded off and ejected into the parasitophorous vacuole (Fig 2) In a number of organisms a few of the micronemes were retained and a few rod-like structures similar to the micronemes were observed in the later stages of development. The trophozoite was limited by a single unit membrane and became more ellipsoidal as it grew in size. At this stage two nucleoli were often observed within the single nucleus.

As the trophozoite developed into the schizont the continued growth of the cyto-


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plasm was accompanied by a number of nuclear divisions. During nuclear division an eccentrically located nuclear spindle was present (Figs. 8 & 10). The nuclear poles (centrocones) had a dense matrix from which microtubules radiated into the nucleoplasm (Fig. 8). The outer nuclear membrane enclosed the nuclear poles and the nuclear membranes retained their integrity during nuclear division. At this stage centrioles were observed in the cytoplasm close to the nuclear poles (Fig. 8). The centriole was composed of nine single microtubules arranged in a circle around a single central microtubule (Fig. 9).

The early schizont possessed cytoplasm containing a number of mitochondria, Golgi bodies, multi membraneous vacuoles, and varying amounts of rough endoplasmic reticulum (Fig. 4).

The multi membraneous vacuoles were observed towards the interior of the schizont (Figs. 4 & 7). They contained amorphous ribosome-free material and the limiting layer consisted of a number of closely applied unit membranes (Figs. 15 & 16).

The schizont was enclosed by a unit membrane and possessed a few micropores. The micropores appeared as cylindrical invaginations of the limiting membrane with collars of dense material close to the cell surface (Fig. 5). The micropores observed in the schizont were up to 455 nm deep with an inner diameter of 95 nm and a collar diameter of 155 nm.

At this stage of early schizogony the nuclei were positioned close to the limiting membrane of the organism and it was at this developmental stage daughter formation (merozoite formation) was initiated (Fig. 7). A number of osmiophilic plaques were formed →

Fig. 4. A montage of an early 1st generation schizont. At this stage the cytoplasm of the organism contains numerous nuclei, a refractile body swollen Golgi bodies, and a number of multi-membraneous vacuoles. × 8,000.

Fig. 5. This micrograph illustrates a micropore formed by an invagination of the limiting membrane of the organism. A collar of dense material is present around the neck of the invagination (arrow). × 80,000.

Fig. 6. A portion of a 1st generation schizont showing an intra-vacuolar fold coating the limiting membrane of the schizont. × 90,000.

Fig. 7. An early 2nd or subsequent generation schizont illustrating the dense plaques in the peripheral cytoplasm (arrows) which represent the initiation of daughter (merozoite) formation. × 15,000.

Fig. 8. An enlargement of part of the schizont in Fig. 4 showing a dividing nucleus with its eccentrically located nuclear spindle in which a number of microtubules can be seen. Centrioles are present in the cytoplasm close to the nuclear poles (arrows). × 45,000.

Fig. 9. A cross section of a centriole with the nine microtubules (arrows) arranged in a circle around a single central microtubule. × 90,000.

Fig. 10. A portion of a developing schizont in which it is demonstrated that the two nuclear poles of a dividing nucleus, at this stage of daughter formation, are each directed towards a developing merozoite (ME 1 & 2). × 50,000.

Fig. 11. A later stage of schizogony than that illustrated in Fig. 10. From this micrograph a nucleus is seen to divide between two developing merozoites. The developing merozoites each contain a conoid, rhoptry precursors, a few micronemes, and a swollen Golgi body. × 15,000.

Fig. 12. Part of a schizont showing a dense plaque (arrows) with pieces of unit membranes and underlying microtubules. Note the nuclear pole directed towards the plaque. × 90,000.

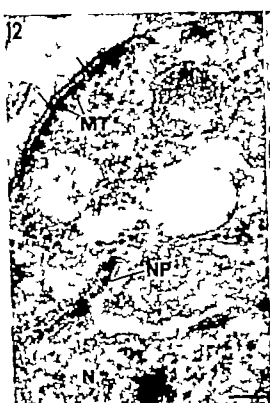
Fig. 13. A cross section through a developing merozoite showing the inner layer of the incompletely formed pellicle. The inner layer consists of a number of individual membrane patches (arrows) each associated with two microtubules. × 90,000.

Fig. 14. A schizont with partially formed merozoites attached to a large mass of cytoplasm. Note the large mitochondrion in the juxta-nuclear cytoplasm of one of the merozoites. × 15,000.

Fig. 15. A portion of the cytoplasm of an early schizont (cf. Figs. 4 & 7). This field is a higher magnification of two multi-membraneous vacuoles containing amorphous, ribosome free material. × 30,000.

Fig. 16. An enlargement of part of Fig. 15 showing the multi-membraneous nature (arrow) of the border of these vacuoles. × 90,000.

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9
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C
NP
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ME2
10
C
RP
NP
M
N
NP
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11


12
MT
NP
N


13

MV
14
G
MI
16

just below the limiting membrane and within their matrices portions of unit membranes developed (Fig 12) A number of microtubules appeared below the plaques (Fig 12) The plaques and microtubules eventually became the inner layer of the pellicle and the sub-pellicular microtubules of the new merozoites, respectively The conoid of the daughter organisms also appeared to be formed in the central region of the dense plaques (Fig. 10) The plaques were seen closely associated with the nuclei. Golgi bodies, which always appeared dilated were usually located in the regions between the nuclei and the plaques (Fig 10) The formation of merozoites seemed to continue as a protrusion of these plaque areas into the parasitophorous vacuole (Fig 11) These protrusions remained coated by the limiting membrane of the schizont which, eventually became the outer membrane of the merozoite's pellicle. At this stage the final nuclear division occurred in such a manner that each of the two poles of the dividing nucleus was directed towards a developing daughter (Fig 10) As the daughter organisms continued to protrude the nuclear poles moved towards and entered them (Fig 11) The nucleus finally divided into two with each portion entering a daughter cell. The nuclear pole was directed towards the anterior of the developing merozoite and was retained until merozoite formation was completed (Fig 17)

Prior to the completion of this nuclear division, a number of membrane bound vesicles (normally 2) were observed in the cytoplasm between the nuclear pole and the conoid, close to the Golgi body (Fig 11) Most likely these vesicles constitute the precursors of the rhoptries of the merozoite. A few dense rod-like structures, probably representing the first of the micronemes, were also present Other cytoplasmic organelles such as mitochondria, centrioles, rough endoplasmic reticulum, and ribosomes were also observed within the immature merozoites after completion of nuclear division (Fig 11)

The inner layer of the pellicle of the immature merozoites seemed to be incompletely formed. This inner layer was first noticed as a number of patches of dense material within which small portions of unit membranes could be distinguished. In cross sections of the developing merozoites it was observed that these patches were situated round the periphery and each patch was associated with two microtubules (Fig 13) Apparently these areas fuse and form the inner layer of the complete pellicle As the daughters matured, the number of micronemes in the apical cytoplasm increased (Fig. 19) and the contents of the vacuoles or rhoptry precursors became more osmiophilic At this stage ducts of the rhoptries were demonstrable and they seemed to be associated with the conoid (Fig 17) Thus the formation of the club-shaped rhoptries was completed An increase in the length of the merozoites was accompanied by a reduction in the size of the residual cytoplasmic mass of the schizont (cf Figs. 14 & 19) Finally the mature merozoites were released from the small residual mass of cytoplasm. Some of them contained a number of polysaccharide granules in their cytoplasm (Fig 19)

Generation Differences

Schizonts were observed in all the examined portions of the small intestine of the infected chickens. This form of the parasite was the only one present up to 96 hours p.i., whereas by 108 hours p.i. both schizonts and gameto-

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Fig 17 An immature merozoite in which a nucleus with a nuclear pole is present. Note also the rhoptry with the duct (arrow) directed towards the conoid. × 30,000

Fig 18 Part of a fully formed merozoite showing the conoid, some micronemes and mitochondria. Note the normal appearance of the Golgi body × 30,000

Fig 19 A micrograph of a mature schizont which illustrates how the fully formed merozoites are attached to a small residual mass of cytoplasm. Each merozoite possesses a conoid, a nucleus with a nucleolus, some rough endoplasmic reticulum, some mitochondria and few polysaccharide granules. × 15,000





RM
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19

cytes were present. At 120 and 144 hours p.i. schizonts were rarely found. From the observations of *Boles & Becker* (1954) we know that a number of schizogonic cycles must have occurred. In our ultrastructural study at 48 hours p.i., organisms were observed which showed examples of all the different stages in the development of the 1st generation schizonts, i.e. all forms from newly entered sporozoites (Fig. 1) to mature schizonts with numerous merozoites were present. Consequently, since no synchronized development occurred, the schizonts observed in chickens killed after longer p.i. periods do not belong to one and the same generation but will be representatives of a mixture of generations. However, the process of schizogony undergone by the parasite was the same in all the chickens examined.

1st generation schizonts. The 1st generation schizonts could be identified by the presence of the so-called refractile body which is present in the infecting sporozoite (Fig. 1) and which persisted until a late stage in the development of the schizonts. This structure disappeared with the 1st generation schizonts and no evidence of any refractile body was ever observed in the 1st generation merozoites. This generation of schizonts could also be characterized by their large size (Fig. 4) and the large number of merozoites produced. As many as 65 merozoites were observed in a single section through a mature schizont. In addition, the surface area of the late schizont, in certain organisms, was increased by deep invaginations of the limiting membrane.

It was also observed that the intra-vacuolar folds in the parasitophorous vacuole were very extensive in the 1st generation schizonts so that large areas of the developing parasites seemed to be coated with these folds (Fig. 6). The intra-vacuolar folds were much less extensive in the later generations of schizonts and gametocytes.

2nd and subsequent generations. As stated previously, it was not possible to differentiate between organisms of these later generations on a time basis. After the 1st generation, individual organisms did not possess any features which were characteristic for the generations to which they belong. They all differed from the 1st generation of organisms by being smaller, producing fewer merozoites, and lacking a refractile body (cf. Figs. 4 & 7).

DISCUSSION

The host/parasite relationship between *E. brunetti* and the epithelial cell is similar to that reported for other *Eimeria spp.* (*Scholtyseck* 1973 a). Although the nutritional aspects of the host/parasite relationship are not clearly understood for eimerian parasites, it is believed that the intra-vacuolar folds are involved with parasite nutrition (*Scholtyseck* 1973 a). The folds could be functioning either by breaking off and degenerating to provide a large pool of raw materials for parasite use or by aiding the transport and exchange of material between the host cell and parasite via the parasitophorous vacuole. Either of these suggested functions could explain the more extensive folds observed for the large 1st generation schizonts which will have greater nutritional needs than the smaller schizonts and gametocytes. Thus, the extensive folds could be a reflection of the increased metabolic requirements at this stage of development.

The morphology of the host/parasite relationship of *E. brunetti* differs from that observed for *Isospora felis* and *I. revolta* (*Pelster* 1973) and *Toxoplasma gondii* (*Sheffield* 1970, *Pelster & Piekarski* 1972, and *Ferguson et al.* 1974). In these species the parasitophorous vacuole was limited by a thick layer which lacked intravacuolar folds.

The form and extent of dedifferentiation in the infecting organism was identical and independent of the duration of infection. It is thus impossible to determine whether the organism is going to undergo sexual or asexual development.

Our observations on the intracellular dedifferentiation of the infecting organism are similar to those described by *McLaren* (1969)

for *E. tenella*, and *Lee & Millard* (1971) for *E. praecox*. Although a few micronemes were retained there was no evidence for the retention of other anterior organelles as has been observed in *Toxoplasma* (*Ferguson et al.* 1974 & 75) and *E. ferrisi* (*Chobotar et al.* 1975). The ejection of cytoplasm containing microneme like structures has been reported before for *E. magna* (*Speer et al.* 1973) but in this study of *E. brunetti* sub-pellicular microtubules were also found to be present in the ejected cytoplasm.

The process of nuclear division within the schizont resembled that observed in the microgametocyte (*Ferguson et al.*, in prep.). In addition, the structure of the eccentrically located nuclear spindle is identical to that reported for some *Eimeria spp.* (*Mehlhorn* 1972, *Dubremetz* 1973 and *Hammond et al.* 1973). The structure of the centriole is similar to that observed in *E. necatrix* (*Dubremetz* 1973) and thus differs from the classical description of centrioles in animal cells.

The asexual multiplication undergone by the coccidia including those belonging to the family *Sarcocystidae* (*Levine* 1973) has been reviewed by *Hammond* (1973). The process observed in our study is similar to that undergone by the majority of coccidians belonging to the genus *Eimeria*, a process termed ectomerogony by *Hammond* (1973).

A similar process of asexual multiplication has been described for the other *Eimeria spp.* infecting chickens (*Dubremetz* 1975, *Fernando* 1974, *Fernando & Stockdale* 1974, *Lee & Millard* 1971, *McLaren* 1969, *Melhorn et al.* 1972 and *Sénaud & Černá* 1969). In the present study no evidence was found for the presence of sporozoite-shaped schizonts or for the internal initiation of daughter formation as has been described for *E. callospermophili* (*Roberts et al.* 1970 a & b). According to *Scholtyseck* (1973 b) a characteristic of the coccidia is that daughter formation is associated with the final nuclear division; our observations tend to confirm that this proposal also holds true for *E. brunetti*.

The multi-membraneous vacuoles observed in our study have the same substructure and are thus probably synonymous to the structures termed "Golgi adjuncts" by *Ogino & Yoneda* (1966). These vacuoles have been observed during asexual development in a *Toxoplasma* and a number of *Eimeria spp.* (*Ogino & Yoneda* 1966, *Sheffield & Melton* 1968, *Sampson & Hammond* 1972, *Roberts et al.* 1970 a and *Müller* 1975). They have also been observed in the developing macrogamete of *E. magna* (*Speer et al.* 1973). We did not find any relationship between these vacuoles and the Golgi body and therefore we use the term multi membraneous vacuoles which is similar to the term ("Vacuole plurimembranaire") used to describe similar vacuoles in *E. necatrix* (*Dubremetz* 1975). The function of the vacuoles is unknown but they are only present in actively developing organisms. The multi-membraneous vacuoles have a lysosome like morphology and it may be possible that they are functioning as lysosomes and are thus aiding in the recycling of material during development.

The development of the inner layer of the pellicle of the merozoite from a number of dense patches under the limiting membrane resembles that described for *E. necatrix* (*Dubremetz* 1975) but in that organism there were extensions into the cytoplasm of the merozoite, whereas such extensions were absent in the *E. brunetti* which we examined.

In the mature merozoite the Golgi body has a less dilated and a more normal appearance than in the schizonts and developing merozoites (cf. Figs. 7 & 18). This could indicate that the dilation of the Golgi body in the latter two groups is a result of a participation of this organelle in the synthesis of precursors or building material for the developing merozoites.

It has been reported for *E. contorta* (*Müller* 1975) that the mature merozoites migrate out of the parasitophorous vacuole through and out of the host cell into the intestinal lumen. No evidence for such an active process was observed in our ultrastructural study and from light microscope examination of toluidine blue stained sections it also appeared that the merozoites played a passive

role waiting for the natural degeneration and lysis of the host cell.

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AN ENTEROTOXIN PRODUCED BY *CLOSTRIDIUM PERFRINGENS* TYPE D PURIFICATION BY AFFINITY CHROMATOGRAPHY

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Uemura, T & Skjelkvåle R. An enterotoxin produced by *Clostridium perfringens* type D Purification by affinity chromatography Acta path. microbiol scand. Sect. B, 84 414-420 1976

Clostridium perfringens type D 9867 produced enterotoxin immunologically identical to that produced by types A and C which are responsible for *C perfringens* food poisoning. Enterotoxin from type C 3386 and type D 9867 was produced in Duncan og Strong sporulation medium (DS medium) The supernatant fluid from DS-cultures was used for purification of the enterotoxin by affinity chromatography on a monospecific anti-enterotoxin-coupled CNBr activated-Sepharose 4B and activated CH-Sepharose 4B column. The enterotoxin purified by this one-step procedure proved to be of a purity comparable to that obtained by conventional methods, and possessed lethal activity in mice.

Key words Enterotoxin *Clostridium perfringens* affinity chromatography

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On the basis of production of four major lethal toxins, *Clostridium perfringens* is differentiated into five types, A to E. Only types *A and C have been implicated in food poisoning outbreaks* (14) The symptoms of the illnesses caused by each type are easily distinguishable The beta-toxin produced by type C is supposed to be responsible for the characteristic and severe symptoms of enteritis necroticans (19)

The classical *C perfringens* food poisoning is caused by certain strains of type A which are able to produce an enterotoxin. The predominant symptoms are diarrhea and abdominal cramps (5) Several investigators have contributed to increase the understanding of the nature of type A food poisoning The enterotoxin has been purified from sporulated cells, characterized biologically and physicochemically (13 4 9) shown to be *responsible for the diarrhea (15 17)* detected in diarrheal faeces obtained from experimental (17) and from genuine cases of food poisoning (12)

Most type A strains isolated from faecal samples obtained from cases of food poisoning produced enterotoxin in *Duncan & Strong* sporulation medium (DS medium) (3) but few strains from natural sources were enterotoxigenic (20) These results confirm the importance of the enterotoxin positive strains of type A in the *C perfringens* food poisoning

Skjelkvåle & Duncan (11) demonstrated

that type C strains isolated from cases of enteritis necroticans in New Guinea produced enterotoxin, biologically and immunologically identical to that of type A. This indicates that the production of enterotoxin cannot be restricted to type A.

In this paper the enterotoxin production by a type D strain is demonstrated, enterotoxin from the culture supernatant fluid of types C and D is purified by affinity chromatography and the problems involved in the enterotoxin formation by *C. perfringens* in connection with food poisoning are discussed.

MATERIALS AND METHODS

Strains

C. perfringens type A, NCTC 8239 type C, 5386 were obtained from *C. L. Duncan*, Food Research Institute, University of Wisconsin, Madison, Wisconsin. Nine strains, consisting of one type B and eight type D were kindly provided by *O. Sandvik*, National Veterinary Institute, Norway. One strain type D 9867 was made available by L. D. S. Smith, Virginia Polytechnic Institute and State University, Blackburg, Virginia.

Heat-treatment

A 0.5-ml portion of each stock culture of different types of *C. perfringens* in cooked meat medium (Difco Laboratories, Detroit, Michigan) was inoculated into 10 ml of newly prepared cooked meat medium, which was heated for 20 min at 75 C in a water bath and incubated for 18 to 24 h at 37 C. This heat-treatment followed by incubation of organisms in cooked meat medium was repeated five times. Finally 1 ml of the culture was transferred into 10 ml of fluid thioglycolate medium (Difco) followed by incubation at 37 C for 12 to 18 h. One millilitre of the culture was inoculated in 100 ml of DS medium (3) and incubated for 24 to 30 h at 37 C. The DS culture was centrifuged at 10,000 × g for 15 min and the supernatant fluid was subjected to the test for enterotoxin production. Single-colony isolations were made from the cultures which showed enterotoxin production and each isolate was typed according to the method of *Oakley & Warrack* (8) using C. perfringens diagnostic serum (Wellcome Laboratories, Beckenham). The isolates were maintained in cooked meat medium at room temperature and used for enterotoxin production in DS medium; the supernatant fluid was subjected to affinity chromatography for purification of the enterotoxin.

Reference Enterotoxin and Anti-enterotoxin

Enterotoxin used as reference was purified from the extract of sporulating cells of *C. perfringens* type A strain 8239 grown in DS medium for 8 h at 37 C. The procedures for purification of enterotoxin and production of rabbit immune serum have been described (9). Monospecific anti-enterotoxin was purified by affinity chromatography as described below.

Affinity Chromatography

Activated CH-Sepharose 4B and CNBr activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala) were used as immuno-adsorbent resin; purified enterotoxin or mono-specific anti-enterotoxin as coupling substance. For coupling, $NaHCO_3$ buffer (0.1 M, pH 8.0) was used. The buffer-change for anti-enterotoxin was done on a PM 10 membrane (Amicon Corp., Lexington, Mass.) in an ultrafiltration chamber by repeated addition of the coupling buffer and concentration by high pressure N_2-gas, alternately.

One gram of dry gel was swollen followed by washing on a sintered glass filter with 200 ml of 1 mM HCl. The slurry was transferred to a screw cap test tube which contained 15 mg of anti-enterotoxin or of purified enterotoxin in 10 ml of the coupling buffer. The mixture was rotated end over end for 18 h at 4 C, filtered to remove uncoupled anti-enterotoxin or enterotoxin, washed with 50 ml of coupling buffer, and the gel was treated with 50 ml of ethanolamine (1 M, pH 9.0) for two h. The anti-enterotoxin or enterotoxin coupled Sepharose was washed three times each with 50 ml carbonate buffer (0.2 M, pH 8.0 with 0.5 M NaCl) and acetate buffer (0.. M, pH 4.0, with 0.5 M NaCl), equilibrated in Tris buffer (0.1 M, pH 8.0, with 0.5 M NaCl) and packed in a column (K9/15, Pharmacia).

In order to purify monospecific anti-enterotoxin, enterotoxin-coupled CNBr activated Sepharose 4B column was used. Twenty millilitres of rabbit antiserum was dialyzed against 0.2 M Tris buffer (pH 8.0, with 0.5 M NaCl) and loaded on the column. The column was washed with 50 ml of the buffer and adsorbed anti-enterotoxin was eluted with 0.2 M glycine buffer (pH 2.3, with 0.5 M NaCl).

For purification of enterotoxin, anti-enterotoxin-coupled CNBr activated-Sepharose 4B and activated CH-Sepharose 4B column was used. To 60 ml of the supernatant fluid containing enterotoxin, 2.3 g of NaCl was added, allowed to stand overnight and loaded on the affinity column. Elution of enterotoxin from the immuno-adsorbent column was made by buffer solutions in the following order; 10 ml of phosphate buffered saline, 10 ml of phosphate (0.02 M, pH 7.0), 10 ml of 0.01 N NaOH in 0.85 per cent NaCl, 50 ml of acetate (0.2 M

pH 2.3 with 0.5 M NaCl) and 50 ml of Tris (0.2 M, pH 8.0, with 0.5 M NaCl) One millilitre fractions were collected into 1 ml of phosphate buffer (0.2 M, pH 7.0) as described by *Scott & Duncan* (10) Protein and enterotoxin contents of each fraction were determined.

Protein Determination

Protein content was measured by the method of *Lowry et al.* (7) using bovine serum albumin (BSA) (Armour and Co., Chicago, Ill.) as a standard.

Reversed Passive and Passive Haemagglutination Tests

Anti-enterotoxin potency was determined by passive haemagglutination (PHA) test according to the procedure described elsewhere (16)

Enterotoxin was quantitated by reversed passive haemagglutination (RPHA) test coupling of anti-enterotoxin to formalin-treated sheep erythrocytes was performed as described previously (18) The haemagglutination pattern with 1 ng/ml of the reference enterotoxin purified by conventional methods (9) was read as the end point of the RPHA reaction. Culture supernatants were diluted 1:80 followed by twofold dilution with phosphate buffered saline (PBS) containing 0.25 per cent BSA. By this method, the minimum detectable level of enterotoxin in the supernatant fluid was approximately 0.08 μg/ml

Polyacrylamide Disc Gel Electrophoresis

The electrophoresis was performed as described by *Davis* (1) The gels were stained with Coomassie brilliant blue G-250 and scanned by a digital computing densitometer type DCD-16 (Gelman Inst. Co Ann Arbor Michigan)

Agar Gel Double Diffusion Test

Agar gel double diffusion test was done as described by *Sakaguchi et al.* (9)

Mouse Test

Biological activity of the purified material was tested using mice weighing 20 to 25 g, as described elsewhere (9)

RESULTS

Purification of Anti-enterotoxin

Sixteen milligrams of monospecific anti-enterotoxin was purified from 20 ml of rabbit antiserum against enterotoxin by affinity chromatography on a CNBr activated Sepharose 4B column to which 13 mg of purified enterotoxin produced by *C perfringens* type A strain 8239 had been coupled. The elution patterns are shown in Fig. 1

Selection of Enterotoxin positive Strains

Ten strains of types B and D were heat treated and the enterotoxin production in DS media was tested by RPHA test. After five times repeated heat-treatment type D strain 9867 produced enterotoxin, 1.3 μg/ml None

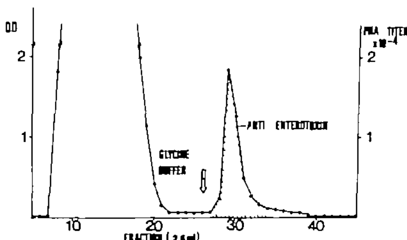


Fig 1 Affinity chromatography of anti-enterotoxin antibody on an enterotoxin-coupled CNBr activated-Sepharose 4B column. Twenty millilitres of rabbit antiserum against purified *C perfringens* enterotoxin were applied.

TABLE 1 *Purification of* C. perfringens *Enterotoxin by Affinity Chromatography on an Activated CNBr-Sepharose 4B Column*

Type	Sample	Volume ml	Protein mg/ml	Protein Total mg	RPHA titre /ml	RPHA titre Total	Specificity (RPHA titre/mg protein)	Fold purification	Recovery in RPHA
…ain …86	Supernatant fluid	80	15	1,200	25,600	2,048,000	1 700	1	1
	Affinity effluent	6		0 74		480,000	650 000	382	0.23
D …rain …67	Supernatant fluid	80	15	1,200	8,000	640 000	533	1	1
	Affinity effluent	8		0.43		173,000	402,000	750	0.27

of the other strains of type B and D produced enterotoxin after five and ten times repeated heat-treatment. Enterotoxin positive DS-culture of strain 9867 was streaked on TSN agar (BBL) and incubated anaerobically at 37 C for 24 h. Four colonies were picked from the agar and subjected both to typing and enterotoxin tests. All four isolates came out as type D by animal protection tests and produced enterotoxin, ranging from 1.3 μg/ml to 8 μg/ml. Subjected to further heat-treatment, no significant increase in enterotoxin production was seen.

Purification of Enterotoxin by Affinity Chromatography

The supernatant fluid of type C strain 5386 and type D strain 9867 grown in DS medium was filtered through a column containing anti-enterotoxin-coupled activated CH-Sepharose 4B or CNBr activated Sepharose 4B. The column was used repeatedly five times. The results are summarized in Table 1. Expressed in RPHA titre, recovery rates were 23 per cent and 27 per cent, respectively and the specific activity increased 382 and 750-fold. When activated CH-Sepharose 4B was used for the affinity chromatography significant differences in these titres were not seen.

The elution patterns obtained by affinity chromatography from types C and D are

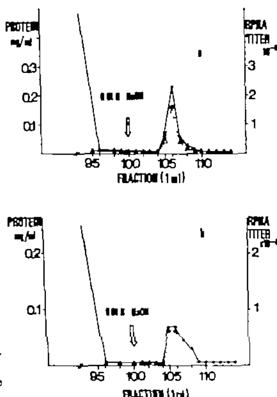


Fig. 2. Affinity chromatography of *C. perfringens* enterotoxin on an anti-enterotoxin-coupled CNBr activated-Sepharose 4B column. Eighty millilitres of the supernatant fluid of DS culture of *C. perfringens* type C 5386 ( ) and type D 9867 (b) was applied.
O—O: protein.
Δ—Δ RPHA titre.

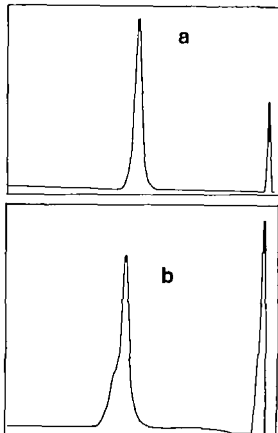


Fig 3 Acrylamide gel electrophoresis of enterotoxin purified by affinity chromatography Twenty micrograms of the enterotoxin was run. Type C 5386 (a) and type D 9867 (b)

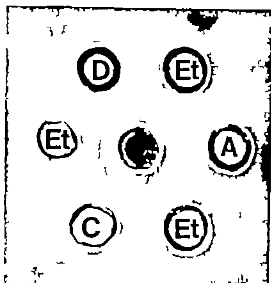


Fig 4 Agar gel immunodiffusion test. Center well received rabbit antiserum against purified enterotoxin from *C perfringens* type A. A, C and D wells received enterotoxin purified by affinity chromatography from the supernatant fluid of type A NCTC 8239 type C 5386 and type D 9867 respectively (100 μg/ml) Et, enterotoxin purified by conventional method (50 μg/ml)

shown in Fig 2 a and Fig 2 b. Two peaks emerged enterotoxin was detected in fractions after NaOH-elution (the second peak) Affinity chromatography on an activated CH-Sepharose 4B column showed the same patterns.

Purity and Identity of Enterotoxin

Purity of the purified material was examined by polyacrylamide disc gel electrophoresis. Fig 3 a shows the scanning patterns of enterotoxin purified from type C, and Fig 3 b from type D

After electrophoresis on disc acrylamide gels of different concentrations the purified enterotoxin from type C showed only one band. The purified material from type D was resolved into one major and one minor protein band as seen from the shoulder in the densitometric tracing (Fig 3 b) The minor band was determined by digital computing densitometer to contain 6 per cent of the total amount of protein. It has been reported that similar minor band(s) may be seen in purified enterotoxin from type A, (6 13) and this may be due to modification of the enterotoxin. Similar results were obtained when type D supernatant fluid was run on anti-enterotoxin-coupled activated CH-Sepharose 4B column.

The purified materials were also examined by agar-gel double immunodiffusion test Fifty micrograms of purified protein gave a single band against antiserum prepared against purified enterotoxin from type A (Fig 4) These results establish the immunological identity of enterotoxin purified from different toxigenic types of *C perfringens*

Ten micrograms of the purified material from types C and D were lethal to mice

DISCUSSION

Monospecific anti-enterotoxin was purified by affinity chromatography on a purified enterotoxin-coupled CNBr activated-Sepharose 4B column. In preliminary experiments, affinity chromatography was run with 2 ml portions of rabbit antiserum, but the recovery in PHA titre (anti-enterotoxin) did not exceed 25 per cent. When the application volume of the serum was increased up to 20 ml, highly concentrated anti-enterotoxin was eluted (Fig 1) but recovery still remained as 23 per cent. Sixteen milligrams of anti-enterotoxin were purified by one-run, and this monospecific anti-enterotoxin was used for coupling the affinity resin.

To simplify the procedure, activated CH Sepharose 4B and CNBr activated-Sepharose 4B were preferred as affinity chromatography resins. These reagents are purchased as dry powder and need no coupling reagent like carbodiimide. Desorption of antigen from the immunoadsorbent column is usually done by exposure of low or high-pH buffer. The antigenicity of enterotoxin was affected when exposed to pH higher than 10.5 or lower than 4.5 and exposed to pH 11 for 15 min 75 per cent of enterotoxin in RPHA titre was destroyed (17). *Scott & Duncan* (10) first succeeded in desorbing enterotoxin from the immunoadsorbent column by elution with 0.01 N NaOH and purified the enterotoxin from partially purified material though a great part of the biological activity was lost. According to their data, enterotoxin did not desorb from the immunoadsorbent column when the pH of the NaOH decreased from 11 to 9.9. In this work, therefore 0.01 N NaOH was used as elution buffer.

Enterotoxin is synthesized by *C. perfringens* during sporulation. All investigators have started the purification of enterotoxin from crude cell extract prepared by sonic treatment of young sporulating cells (6, 13, 9). Most of the enterotoxin is released into the medium by rupture of sporulated cells during prolonged incubation (7, 9). For affinity chromatography the laborious production of cell extracts could be omitted. In this work the supernatant fluid from overnight-culture of *C. perfringens* in DS medium was applied to the affinity column to which NaCl had been added at 0.5 M to eliminate non-specific adsorption to the column. In the affinity system mentioned above neither sonic treatment, extraction procedures, nor gel filtration was necessary and only one peak emerged after elution with 0.01 N NaOH. Thus the specific activity increased 750-fold in one-step purification.

Strains isolated from food poisoning outbreaks exhibit considerable variation in enterotoxin production on artificial media. Type C 5386 is a strong enterotoxin-producing strain (Table 1) and sporulates readily in DS medium. Type D 9867 shows lower enterotoxin production, which is likely to be due to its lower sporulation rate in DS-medium.

In this investigation the strains were assayed for enterotoxin potency after heat treatment which might enhance the toxin formation (11, 18). Results from *in vitro* experiments do not always indicate the real situation *in vivo*. Because enterotoxin may have to be produced in the intestine to cause diarrhea, many factors influence enterotoxin production *in vivo*. The results of this study indicate that some type D-strains may have potency to cause food poisoning of the same type as that caused by some type A strains. Little information is available about enterotoxin production by types other than type A. Even among type A strains, when isolated from natural sources, the number of enterotoxigenic strains are few (20). The results of this study establish the need for enterotoxin production test for type A as well as for other toxigenic types when food poisoning outbreaks occur. These results suggest that the toxin produced by type D may possess the same characteristics as those described to be produced by enterotoxin from types A and C.

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CORRELATION OF THE CHARACTERS FERMENTATION OF TREHALOSE, NON TRANSMISSIBLE RESISTANCE TO TETRACYCLINE, AND RELATIVELY LONG FLAGELLAR WAVELENGTH IN *PROTEUS MORGANII*

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Siboni, K. Correlation of the characters fermentation of trehalose, non-transmissible resistance to tetracycline, and relatively long flagellar wavelength in *Proteus morganii*. Acta path. microbiol. scand. Sect. B, 84 421–427 1976.

An investigation of strains of *Proteus morganii* isolated from patients in Odense, Denmark, and of 21 serotype strains revealed a close correlation between the fermentation of trehalose and a non-transmissible resistance to tetracycline. The trehalose fermenting, tetracycline resistant strains had a longer flagellar wavelength than the non-fermenting, sensitive strains Resistance to chloramphenicol occurred only in strains of the former group.

Key words *Proteus morganii* correlation of characters.

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Since the study published by *Thjøtta* (1920) *Proteus morganii* has been considered a heterogeneous group. There has since been some clarification with observations about the different properties of the species

Edwards & Ewing (1972) and Bergey's Manual (*Lautrop* 1974) agree that some of the strains ferment trehalose (tr^+) *Rauss & Vörös* (1959) could further divide their strains into 12 biotypes using fermentation of galactose, raffinose, sucrose, sorbose, and sorbitol.

Leifson et al (1955) observed longer flagellar wavelength in tr^+ strains (mean 2.34 μm, s.d. 0 17 μm) than in tr^- strains (mean 2.14 μm, s.d 0 15 μm) the tr^+ strains were in this respect similar to *Proteus rettgeri* and *Providencia* and the tr^- strains to *Proteus mirabilis* No descriptions of experiments performed in order to induce the formation of trehalase in tr^- strains of *Proteus morganii* are known to the author of the present paper As trehalose is the only known substrate for trehalase (*Nunzawa & Hashimoto* 1970) there is no quick enzymatic test for the presence of trehalase analogous to the o-nitrophenyl-β-d-galactopyranoside test for β-galactosidase (*Bülow* 1964)

Falkow et al. (1964) found 50 per cent

Guanine + Cytosine in their ten strains of *Proteus morganii* but they did not report whether the strains fermented trehalose.

Tomaschoff (1969) and *v. Graevenitz & Nourbakhsh* (1972) regarded *P. morganii* as naturally sensitive to tetracycline, but *Perch* (1954), the first to examine this problem, found four of her 18 serotypes resistant to tetracycline while all strains were sensitive to streptomycin and all but one to sulphonamide. Perch's strains had been isolated before the era of tetracycline and chloramphenicol in Hungary (*Rauss* 1936) and Great Britain (*Cook* 1948) or were from the National Collection of Type Cultures, London.

Hedges et al. (1973) examined 167 strains of *P. morganii* isolated from human faeces in Pretoria (*Coetzee et al.* 1972). 80 strain were resistant to tetracycline. They could transfer 25 R factors to *E. coli* K 12. 17 conferred resistance to tetracycline.

The starting point of the present investigation was the observation in the routine laboratory that most strains of *P. morganii* were sensitive to sulphonamide, tetracycline, streptomycin, and chloramphenicol and resistant to ampicillin and polymyxin B—this in accordance with *Tomaschoff* (1969) while a few strains were resistant to tetracycline and often to chloramphenicol. Inspired from the paper by *Leifson et al.* (1955) the strains tested for fermentation of trehalose: the first mentioned strains were tr⁻ while the latter were usually tr⁺. Some strains had been stored since 1966 and 1968 and in 1975 all strains of *P. morganii* were kept in order to establish whether they could be divided into two groups, one of trehalose fermenters, resistant to tetracycline and with a longer flagellar wavelength, and another one of trehalose non fermenters sensitive to tetracycline, and with a shorter flagellar wavelength.

STRAINS AND METHODS

The strains were isolated on Conradi-Drigalski plates from specimens of blood, pus, sputum, and urine from patients in Odense county mainly in Odense University Hospital.

Twenty-eight strains were kept from 1966 and 1968. One hundred and six strains from 92 patients were isolated 1975 from 51,000 specimens. Only one strain from each patient was included in the study.

For comparison were included Perch's 21 type strains isolated before 1954 (*Perch* 1954) and provided by Dr. *Ida Ørskov*, The International Escherichia Centre, Statens Seruminstitut, Copenhagen. They represent 17 O-antigens and 6 H-antigens.

The media were as described by *Leistrup* (1956) and by *Møller* (1955). The trehalose plates were Conradi-Drigalski plates containing trehalose 0.5 per cent instead of lactose.

All strains included were facultatively anaerobic, reduced nitrate to nitrite, produced indol, urease, and ornithine decarboxylase, but did not produce arginine dihydrolase. They produced H_2S after 3–4 days, but did not liquefy gelatine at 22–24° C within 7 days. They were Voges-Proskauer and oxidase negative. They fermented glucose (+gas) but did not ferment rhamnose, sucrose, inositol, mannitol, or sorbitol within 7 days. The 28 strains isolated in 1966 and 1968 and Perch's 21 type strains fermented galactose (24 h) and glycerol (3 days) but did not ferment raffinose, sorbose, adonitol or erythritol, nor did they hydrolyse esculin and salicin.

Routine tests for sensitivity to antibiotics were performed by the diffusion method using Rosco's tablets (Neosensitabs®, Rosco, DK 2630 Taastrup, Denmark) on the medium described by *Thomsen* (1967). The tetracycline- and chloramphenicol tablets both contained 400 µg. Inhibition zones ≥ 23 mm correspond to IC50 ≤ $2^{1.5}$ = 2.8 µg/ml tetracycline and IC50 ≤ $2^{3.5}$ = 11 µg/ml chloramphenicol. The standard deviation of the inhibition zones in repeated experiments for tetracycline was 1.2 mm and for chloramphenicol 2.0 mm.

Additional Tests

All tr⁺ T-R (tetracycline resistant) strains (8) and selected tr⁺ T-R and tr⁻ T-S (tetracycline sensitive) strains representing the different resistance types, a total of 37 strains, were the objects of additional investigation.

Tests for sensitivity to kanamycin and gentamycin by the diffusion method: all the strains were sensitive.

Tube dilution tests for sensitivity to tetracycline and chloramphenicol were performed in two-fold dilution series of the antibiotic in infusion broth, 2^{10}–2^{-5} µg/ml (1,000–0.032 µg/ml), two tubes per dilution, 0.5 ml per tube. Inoculum: one loopful overnight broth culture diluted 10^3 containing an average of $10^{4.5}$ bacteria. The end-points were read after 24 and 48 h, and IC50 was determined by *Kärber*'s method (1931). IC50 × 1.2 = Minimum Inhibitory Concentration. Each experiment included *E. coli* K 12 strain 7458/41 as a reference

log of mean IC50 for tetracycline for this strain was 0.23 (1.17 µg/ml) s.d. = 0.46 log_2, i.e. 0.5 dilution step.

Transfer experiments were performed as simple matings with *E. coli* K 12, F⁻ strain W 3132, received from Dr *Id. Ørskov* Statens Seruminstitut, and made resistant to nalidixic acid 256 µg/ml (*Søgaard* 1973). The selection was performed on Conradi-Drigalski plates containing nalidixic acid 25 µg/ml and tetracycline 10 µg/ml or chloramphenicol 20 µg/ml.

Induction of trehalose production was attempted in two ways: a) by the addition to cultures in trehalose media of cyclic adenosinemonophosphate (Adenosin-3',5'-cyclophosphat no. 24,801 Merck, Darmstadt) dissolved in Tris-buffer 50 mM, pH 7.4–7.5 (Sigma Chemical Company, St. Louis). The final concentration was c. 1.2 × 10^{-4} M (*Pastan & Perlman* 1970); b) plating the strain on a trehalose plate with a tetracycline tablet.

Selection of tr T-R mutants from strain 660 III/73 tr T-R (isolated in Odense 1973) and from Perch's strain F 5706 tr^+ T-R, was performed with the penicillin treatment technique for isolation of auxotrophs described by *Miller* (1972) in Hauser's minimal medium with trehalose 2 per mille (*Helding & Collet* 1971).

Staining and measurement of flagella: After addition of 1/3 volume phosphate buffer pH 7.5 to an overnight broth culture the bacteria were washed twice in distilled water. The staining was as described by *Leifson* (1960); formalin was not used.

For the measurement of flagellar wavelength was used a Carl Zeiss photomicroscope II: objective 100 ×, optovar 1.25 ×, ocular micrometer 10 ×; total magnification: 1250 ×.

Each division of the ocular micrometer was equal to 0.8 µm. Ten flagella of normal curvature on separate organisms were measured, a total of c. 20 wavelengths per strain. This is in accordance with *Leifson et al.* (1955).

RESULTS

Trehalose was the only carbohydrate useful in the biotyping of the strains of *Proteus morganii* investigated.

The 120 strains were resistant to polymyxin B (5 exceptions) and to ampicillin (2 exceptions) and they were sensitive to carbenicillin (1 exception). The results of sensitivity tests with sulphonamide, tetracycline, streptomycin, and chloramphenicol are shown in Table 1.

The 21 trehalose fermenting strains were resistant to tetracycline and 13 of them also to chloramphenicol, while among 99 trehalose negative strains only seven were resistant to tetracycline and two to chloramphenicol. Resistance to sulphonamide and to streptomycin was not more frequent among the trehalose fermenting (tr^+) strains than among the non-fermenters (tr^-). The diameters of the inhibition zones are reported in detail (Table 1) in order to demonstrate the striking difference between strains resistant to tetracycline (T-R)—hardly any inhibition zone, and sensitive strains (T-S)—large zones.

The results of tube dilution tests for sensitivity to tetracycline of 37 selected strains are shown in Table 2.

In Table 3 the resistance types of all the

TABLE 1. *Sensitivity to Antibiotics in Strains of* Proteus morganii *Isolated 1966–68 and 1975*

Zone diameter mm		Trehalose fermenters					Trehalose non-fermenters				
		≤14	15–22	23–27	≥28	Totals	≤14	15–22	23–27	≥28	Totals
1966–68	Sulphonamide	2	–	2	9	13	1	–	7	7	15
	Tetracycline	11	2	–	–	13	2	–	1	12	15
	Streptomycin	–	2	3	8	13	–	–	–	15	15
	Chloramphenicol	–	10	2	1	13		–	2	13	15
1975	Sulphonamide	1	–	–	7	8	4	1	11	68	84
	Tetracycline	8	–	–		8	5		–	79	84
	Streptomycin	1	–	–	7	8	6		2	72	84
	Chloramphenicol	–	3	2	3	8		2	1	81	84

Zone diameters ≤ 22 mm correspond to IC50 ≥ 2.8 µg/ml tetracycline and IC50 ≥ 11 µg/ml chloramphenicol.

TABLE 2. *Results of Tube Dilution Tests for Sensitivity to Tetracycline (24 h Reading) Correlated to the Results of Diffusion Tests and to the Ability to Ferment Trehalose*

IC50 µg/ml	≧0.125	≧0.25	≧0.5	≧1	≧2	≧4	≧8	≧16	≧32	≧64	Totals
Log_2 IC50	–3	–2	–1	0	1	2	3	4	5	6	
Trehalose tetracycline resistant* in diffusion test	–	–	–	–	1	1	2	8	4	1	17
Trehalose tetracycline resistant* in diffusion test	–	–	–	–	–	–	–	2	4	2	8
tetracycline sensitive§ in diffusion test	1	–	10	–	–	1	–	–	–		12
											37

* Diameter of inhibition zone ≦ 22 mm,
§ Diameter of inhibition zone ≧ 23 mm, compare Table 1

TABLE 3. *Strains of* Proteus morganii *Distributed According to Fermentation of Trehalose and Sensitivity to Antibiotics*

Resistance type	Trehalose fermenters			Trehalose non-fermenters			Odense 1975 totals	*Hedges et al.* 1975
	Perch, 1954	Odense 1966&68	Odense 1975	*Perch* 1954	Odense 1966&68	Odense 1975		
SuSTC	–	2	–	–	–	–	–	44
STC	1	–	–	–	–	–	–	1
SuTC			–	–	–	–	–	1
TC	1	8	3	1	–	3	6	8
SuTS	–	–	1			–	1	11
TS	1	–	–			2	2	–
SuT	–		–		1	–	–	2
T	2	3	4		1	–	4	13
SuS		–	–			2	2	6
S	–	–	–			4	4	–
Su			–	1		1	1	2
A or none			–	14	13	72	72	70
SC			–				–	7
C								2
Totals	5	13	8	16	15	84	92	167

Su resistant to sulphonamide, T resistant to tetracycline S resistant to streptomycin, C: resistant to chloramphenicol, A resistant to ampicillin.

TABLE 4. *Correlation of Trehalose Fermentation and Tetracycline and Chloramphenicol Resistance to Flagellar Wavelength*

Fermentation of trehalose	−				+
Resistance to tetracycline, diffusion test,	0/107		8/8		26/26
tube dilution test, IC50 ≥ 2.8 μg/ml ($2^{1.5}$)	1/12		8/8		17/17
Resistance to chloramphenicol, diffusion test,	0/107		3/8		15/26
tube dilution test, IC50 ≥ 11 μg/ml ($2^{3.5}$)	0/12		3/8		12/17
Flagellar wavelength (μm)					
mean/standard deviation	2.36/0 11		2.55/0 13		2.66/0 15
number of strains	12		8		15
median/range	2.32/2.24–2.61		2.56/2.28–2.71		2.64/2.32–2.88
difference of means		*0.19*		*0 11*	
error of difference		0.059		0.060	
probability P		P<0.1%		5%<P<10%	

strains examined are, therefore, arranged according to the two criteria tr⁺/tr and T R/T-S. In the table are included the results with Perch's strains (*Perch* 1954) and for comparison the figures from *Hedges et al.* (1973)

Table 3 shows

a) that no tr⁺ strain was sensitive to tetracycline (left lower quadrant) and that few tr⁻ strains were resistant to tetracycline (right upper quadrant) The probability—calculated from the Odense strains 1975 and from Perch's strains—that this distribution according to the two criteria could occur by chance is 1.38 × 10^{-8} and 2.95 × 10^{-4} respectively (*Fisher* 1950)

b) the total correlation of resistance to chloramphenicol to that to tetracycline in the Odense strains and in those of *Perch* (1954)

In Table 4 the results already reported are correlated to the results from measurement of the flagella. The mean values of T-S and T R strains were different, but the ranges overlapped.

In the mating experiments with *E coli* K 12 F⁻ the 13 tetracycline resistant strains isolated in 1975 did not confer this property nor did the six strains resistant to chloramphenicol confer this trait.

Production of trehalase was not induced into the eight tr⁻ T R strains by cyclic adenosine monophosphate or by the presence of tetracycline.

tr⁻ T R variants could be selected from strain 660 III/75 tr⁺ T R (frequency 1/194 = 0.005) and from strain F 5706 tr⁺ T R in one of three experiments (frequency 1/84 = 0 01)

DISCUSSION

The high frequency at present of transmissible R factors in strains of *Enterobacteriaceae* isolated from patients, and the fact that such factors could also be isolated from strains of *Proteus morganii* has led some investigators to the belief that *P morganii* as a taxon were *naturally sensitive to tetracycline Among the* 80 tetracycline resistant strains of *Hedges et al.* (1973) only 17 could confer this character the 14 conferring resistance to sulphonamide (Su) streptomycin (S) and chloramphenicol (C) at the same time resistance

to chloramphenicol was conferred only from these 14 strains. It is inherent that 63 (80–17) T R strains did not confer this trait. The figures of *Hedges et al* (1973) are included in Table 3 and it is apparent that the resistance pattern SuTSC, frequent in South Africa, was rare among the strains from Odense 1975 none, nor did it occur in Perch's strains isolated before 1954 If the strains with the patterns SuTSC, TSC, SC, and C are excluded from the South African strains, the distribution of the rest hardly differs from that of the Odense Strains 1975 ($\chi^2 = 9.386$ $f = 3$ $2\% < P < 5\%$)

Resistance to tetracycline was not conferred from the Odense strains isolated 1975 (Table 3) The close correlation tr⁺/T R and tr⁻/T-S therefore, speaks for two genetically stable groups. As the same is true in Perch's strains collected from different parts of the world, this correlation could not depend on two local clones dominating the strains from Odense. The present results agree with the observations of *Odakura et al* (1971) concerning the rare transmission of resistance to tetracycline from *Proteus* strains but of course do not exclude that a plasmid could be involved.

The measurement of flagellar wavelength revealed the same difference between tr⁺ T R and tr⁻ T-S strains as did the original observations of *Leifson et al* (1955) with tr⁺ and tr⁻ strains and with the same standard deviation (Table 4) The systematic difference between the mean values of *Leifson et al.* (1955) and the present ones could depend on the magnification factors differing 1 1 times

The relatively few tr⁻ T R strains, c. 5 per cent, may be loss mutants from the tr⁺ strains, favoured in man, where the resistance to tetracycline may be a condition for the survival of the strain, while the ability to ferment trehalose seems useless. They are similar to the tr⁺ strains in their resistance to tetracycline and, some of them, to chloramphenicol and by their mean wavelength which is significantly longer than that of the tr⁻ T-S strains (Table 4) Such mutants could be selected *in vitro* from two tr⁺ T R strains.

The present paper thus creates a synthesis of Perch's observations on resistance to tetracycline in *P morganii* (1954) and the observations of *Leifson et al.* (1955) on the different wavelengths of tr⁺ and (most) tr⁻ strains. It leads to the division of *P morganii* into two—hardly three—natural groups determination of the G + C per cent could decide if there are in fact two species.

CONCLUSIONS

Proteus morganii can be divided into two groups

Group A consists of strains naturally sensitive to tetracycline and to chloramphenicol, trehalose non-fermenters with a shorter flagellar wavelength mean 2.36 µm (*Leifson et al.* (1955) 2.14 µm) It comprised 79/92 strains and is what is now usually regarded as *P morganii*.

Group B consists of strains naturally resistant to tetracycline and, some of them, to chloramphenicol, trehalose fermenters with a longer flagellar wavelength, mean 2.66 µm (*Leifson et al* (1955) 2.34 µm) This group is more similar to the rest of the *Proteus* species than is group A. It comprised 8/92 strains and was joined by 5/92 strains which did not ferment trehalose but were similar in respect of the other properties.

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FURTHER STUDIES OF TWITCHING *STREPTOCOCCUS SANGUIS* ISOLATED FROM THE HUMAN THROAT. ISOLATION OF STRAINS WITH A NEW ANTIGEN

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α-haemolytic streptococci, classified as *Streptococcus sanguis* with spreading growth due to twitching mobility were isolated from 41 per cent of 121 swab cultures from the human pharynx. Sixty-five out of 70 isolates with spreading growth belonged to Lancefield's group H, while 5 isolates constituted a new serological entity. None of 159 non-spreading α-haemolytic streptococci isolated from the same cultures possessed either of these antigens. An examination of strains of types I, II and I/II described by *Washburn et al.* in the group H antisera employed in the present study indicated that type I and type I/II only differ from one another in the degree of cross-reactivity with anti-type II serum. (The type II antigen was not demonstrated in any of the isolated strains.) We support the opinion that type II should not be considered as *S. sanguis*.

Key words: Streptococcus sanguis; group H streptococci; twitching; fimbriae.

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The isolation of polarly fimbriated strains of *Streptococcus sanguis* with spreading growth due to twitching motility has been reported previously by us (6). The studies presented here were undertaken in an attempt to answer the following questions:

1) How frequently do spreading streptococci occur in human throat cultures?

2) Is *S. sanguis* the only streptococcal representative of the human mouth and throat flora that may exhibit twitching motility?

3) Do all strains of *S. sanguis* isolated from throat cultures exhibit twitching motility?

4) What is the serological relationship of twitching *S. sanguis* to the known serotypes of group H streptococci?

MATERIAL AND METHODS

Throat swabs were cultured on 5 per cent human blood agar plates incubated for about 1 week at 35° C in a closed container with a layer of water at the bottom in order to ensure a humid atmosphere. After 2–3 days of incubation, the bacterial

colonies were inspected daily with a magnifying glass for signs of spreading growth. One or whenever possible, two colonies of spreading and non-spreading α-haemolytic streptococci, if present, were isolated and examined for spreading growth in several subcultures incubated for at least one week.

Strains

The *S. sanguis* strains NCTC 7863 NCTC 7865 NCTC 7868 ("Challis") and NCTC 9124 („Wicky") were received from the National Collection of Type Cultures, Colindale London. Another culture of the "Challis" strain, "Challis-Pakula known to be competent in transformation, was kindly given to us by Dr. *R. Pakula* Toronto. The following *S. sanguis* strains were received from the American Type Culture Collection, Rockville, Maryland: ATCC 10556 (= NCTC 7863) ATCC 10557 (it has been proposed that this strain should not be considered as *S. sanguis* (2 3 4)) and ATCC 10558 (= NCTC 7865) These three strains represent types I, II and I/II respectively described by *Washburn et al.* (9)

In addition to 70 spreading strains and 159 non-spreading α-haemolytic strains isolated from throat swabs as described above, the 19 spreading strains described in our previous paper (6) were also included in parts of the study

Cultural and biochemical methods were as described before (6)

Agar plate microscopy was carried out as described before (5) For the purpose of this examination, bacteria were grown on cystine-haemin-nutrient agar (7) without gelatine.

Antisera. The antisera used were partly those produced for the preceding study against the twitching *S. sanguis* strains 13843 and 14558 and partly new antisera raised in rabbits, as described (6) against strains ATCC 10556 (type I) ATCC 10557 (type II) and ATCC 10558 (type I/II) and against two new twitching strains, 10043 and 10281 which did not react either with the above mentioned antisera or with commercial group H sera.

Two other group H antisera raised against strains "Blackburn and HF 90 A (Perryer") at the Streptococcal Department, Statens Seruminstitut, and currently in use there, were also employed.

Furthermore, commercial grouping sera covering groups A through S were used as well as antisera raised against strains of groups T U and V at the Streptococcal Department, Statens Seruminstitut, Copenhagen.

Precipitation

All strains were tested for interfacial precipitation in group H antisera after HCl-extraction according to the method of Lancefield. The strains with spreading growth were also examined in the antiserum produced against strain 10043 and, finally a number of selected spreading and non-spreading strains were tested for precipitation in the antisera to type I, type II and type I/II strains.

Double Diffusion in Agar (Ouchterlony Test)

A pattern consisting of a central well surrounded by six peripheral wells was used. Antiserum was placed in the central well and HCl-extracts of streptococcal cells grown in Todd-Hewitt broth were placed in the peripheral wells.

RESULTS

Incidence

From 19 out of a total of 121 throat swabs cultured, α haemolytic streptococci could not be isolated, mostly because of over-growth by other organisms. Altogether 70 isolates of streptococci with spreading growth of the same kind as that described in our previous paper (6) were made from 50 swab cultures, *i.e.* from 41 per cent of all swabs examined or from 49 per cent of all cultures yielding growth of α haemolytic streptococci.

Biochemical Reactions

The results obtained with the spreading strains isolated during the course of the present study are entered in Table 1 None of the strains showed any significant deviation from the results obtained in the preceding study (6) *i.e.* even the 5 strains which belonged to a distinct serological entity (the "10043-group" *vide infra*) did not differ biochemically from the rest of the strains. The 159 non-spreading isolates of α haemolytic streptococci were not biochemically examined.

Agar Plate Microscopy

Four strains in the "10043-group gave rise to spreading zones, the micromorphological pattern of which and manner of cell movement was typical of twitching motility (5) while the fifth strain of this group" (strain 12318) appeared to spread only by sliding (5) It is possible, however that pronounced sliding in some cases may prevent the demonstration of an existing potential for twitching motility (5)

TABLE 1 *Cultural and Biochemical Reactions of 67 Twitching Strains of* Streptococcus sangius

Character	Result
Small spherical cells	+
Spreading colonies	+
Even turbidity in broth	+
Glucose	+
Galactose	+
Mannose	+
Lactose	+ (2)
Arabinose	— (8d)
Maltose	+
Sucrose	+
Trehalose	+ (1)
Salicin	+ (1)
Mannitol	—
Inulin	+ (1)
Dulcitol	—
Raffinose	+ (24)
Sorbitol	— (14)
Hippuric acid	—
Aesculin	+ (8)
10 per cent bile	—
4 per cent NaCl	— (4)
6 5 per cent NaCl	— (2)
Polysaccharide in 5 per cent sucrose broth	+
Optochin resistance	+
H-antigen	+ (4)
10043-antigen	— (4)
a-haemolysis	+

+ or — indicates the reactions of the majority of strains. Figures in brackets are numbers of deviating strains.
d = delayed reactions.

Among the rest of the isolates, 5 spreading strains picked at random exhibited twitching motility and two non-spreading strains did not.

While strain ATCC 10556 (as well as strain NCTC 7863) repeatedly produced spreading zones typical of twitching motility strains NCTC 7868 ("Challis") NCTC 9124 ("Wicky") "Challis-Pakula" HF 90 A ("Perryer") ATCC 10558 (as well as NCTC 7863) and also ATCC 10557 never produced any signs of twitching or spreading growth Thus, non twitching and therefore presumably not polarly fimbriated strains of *S sangius* do occur—at least among old laboratory strains.

Precipitation

Among the 70 spreading isolates, 65 originating from 46 swab cultures, gave heavy precipitates with one or more of the group H sera (see Material and Methods)

Five isolates from 4 swab cultures were not precipitated by any of the group H sera employed but formed heavy precipitates with antisera produced against two of these isolates (strains 10043 and 10281) In the present paper these 5 isolates are referred to as the "10043-group" In this connection, the word group is used in its general meaning i.e. a number of bacteria (or things) related *to each other because of certain similarities* and thus, it is not meant to indicate a Lancefield serogroup (see Discussion)

None of the spreading isolates other than those of the "10043-group" were precipitated by the sera raised against strains of the "10043-group"

None of 159 non-spreading isolates were precipitated either by the group H sera or by the two antisera to the "10043-group"

The 5 strains of the "10043-group" were tested in commercial grouping sera covering groups A through S as well as in anti-T anti-U and anti-V sera but results were completely negative.

The strains ATCC 10556 (type I) ATCC 10558 (type I/II) "Wicky" "Challis" and "Challis-Pakula" which reacted strongly with the group H sera were either not at all precipitated, or only faintly precipitated by the two antisera to the "10043-group" Except for late trace reactions, also strain ATCC 10557 (type II) failed to react in these two antisera, as well as in antisera to type I and type I/II. In anti type II serum, only the homologous strain reacted strongly and extracts of type I/II and of type I gave but very faint as well as late occurring precipitates, respectively

Thus the reaction of type II in anti type I/II serum and *vice versa* had the appearance of weak cross-reactions, and the difference between the reactions of type I and type I/II in our anti-type II serum was presumably quantitative rather than qualitative

This presumption was verified in double diffusion experiments using extracts of type I and type I/II against anti type II serum and *vice versa*. The homologous reactions appeared as marked bands of precipitate, whereas bands corresponding to the weak cross-reactions seen in the precipitin tests were not observed.

DISCUSSION

The incidence of twitching streptococci in swab cultures from the human throat is higher than that previously reported (6) namely 41 per cent as compared with 15.5 per cent. Even 41 per cent may be an under estimate because isolation of this kind of streptococcus may be impeded by over-crowding of the cultures, by the presence of fast-growing or swarming or swimming (5) organisms, or for other technical reasons. Thus, the statement in Bergey's Manual (1) about *S sanguis* "Infrequently encountered in saliva and throat specimens" is no longer valid.

Our experience so far gained suggests that twitching motility may be restricted to *S sanguis* because all the twitching isolates were highly similar culturally and biochemically to known strains of this species and it seems therefore justifiable, at least for the time being, to classify all of them as *S sanguis* although antigenic differences were observed. The majority of the spreading isolates, 84 out of 89 reacted with anti-group H sera, but 5 isolates from 4 swab cultures did not react either with anti-group H sera or with the other grouping antisera of groups A through V. These 5 isolates produced a heavy precipitate in antisera raised against two of the strains, and they therefore appear to represent a new hitherto undescribed, serological entity. Studies of the chemical nature of the antigen in question are necessary to decide whether it qualifies as a new Lancefield group antigen.

A comparison of our strains with the Washburn types I II and I/II suggests that those of our strains that are related to type I and type I/II belong to group H. Type II (ATCC 10557 = NCTC 7864) which has been proposed to belong to *S mitior* (2, 3 4) differs serologically as well as biochemically from our group H and "10043-group" strains. The faint and late occurring cross-reactions of type I/II and the "10043-group" in anti-type II serum and *vice versa*, may not involve the group antigen. According to *Rosan* (8) and *Cole et al.* (2) type II does not carry the group H antigen.

Coykendall & Specht (4) who studied DNA base sequence homologies in a collection of strains labelled *S sanguis* found that the strains could be divided into three groups. Groups 1 and 3 showed marked homologies, both within and between the groups, whereas group 2 showed much weaker homologies with the other groups. Among *Washburn's* strains, type I fell into group 3 type I/II into group 1 and type II into group 2 The strains of group 2 differed from groups 1 and 3 in that they failed to split arginine and aesculin and to ferment inulin and salicin. Accordingly *Coykendall & Specht* were of the opinion that the group 2 strains, including *Washburn's* type II should not be considered to belong to *S sanguis*

We would like to support this idea which would help to clear up some of the prevailing confusion about *S sanguis* and to make the species more homogeneous.

In precipitin tests using Lancefield extracts, 84 out of 89 strains behaved uniformly but tube agglutination tests carried out with the strains included in the preceding study (6) and with the present strains indicated serological heterogeneity A determination of the antigens possessed by our strains, like that performed by *Rosan* (8) and *Cole et al* (2) in the case of *S sanguis* however must await further studies.

Cole et al. (2) found fimbriae on some streptococcus strains, mainly on those belonging to *S sanguis*. The illustrations presented suggests that the fimbriae observed differ from those demonstrated by us (6) These authors do not mention twitching or spreading of their strains, but at least one of the strains considered as non-fimbriated (NCTC

7863 = ATCC 10556) was by us found to produce spreading zones by means of twitching motility

The majority of all twitching strains isolated belonged to group H. Even if non twitching group H strains may occur in the human throat, they are at least not frequent, since none of 159 non-spreading α haemolytic streptococci reacted with the anti-group H sera or the antisera to the "10043-group" It seems possible, therefore, that fimbriation might be a prerequisite for the parasitic existence of this organism on the pharyngeal mucous membrane. These problems are under study

Two of the reference strains involved in this study (NCTC 7863 and the synonym ATCC 10556 type I) repeatedly produced colonies with spreading zones characteristic of twitching motility whereas six strains (NCTC 7865 NCTC 7868, NCTC 9124 ATCC 10558, "Perryer" and "Challis-Pakula") never produced spreading growth, and attempts at demonstrating twitching by agar plate microscopy in the strains "Wicky" NCTC 9124) "Challis" (NCTC 7868) and "Challis-Pakula" also failed. Likewise, the type II strain, ATCC 10557 has never shown spreading

It is interesting to note that *Washburn et al.'s* type I strain (ATCC 10556 = NCTC 7863) has preserved its capacity for performing twitching motility throughout 30 years. Whether the six non-spreading group H reference strains were fimbriated and capable of exhibiting twitching motility when first isolated remains a moot question. It does not seem improbable although it remains to be demonstrated that strains of *S sanguis* like strains of *Neisseria* and *Moraxella* may loose their fimbriae Preliminary experiments with these organisms suggest that twitching may be a fairly stable character

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TRANSFORMATION OF TWITCHING STRAINS OF *STREPTOCOCCUS SANGUIS*

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Henriksen, S. D & Eriksen, J Transformation of twitching strains of *Streptococcus sanguis* Acta path. microbiol. scand. Sect. B, 84 433–436 1976.

Ninety-five strains of *S sanguis* 90 of which were twitching, were screened for competence in transformation with DNA from the "Challis" strain Seventy-two strains, 68 of sero-group H and 4 of the provisional group 10043 were competent. Fourteen of the competent strains and all strains which appeared to be incompetent were tested with DNA from 3 other strains. Th 14 competent strains were transformed by all the 3 DNAs. One of the apparently incompetent strains was transformed by autologous DNA only Among 8 reference strains (including ATCC 10557 Type II of *Washburn et al.*) 5 were competent. Three of these did not show spreading or twitching. Among 16 non-spreading strains of alphahaemolytic streptococci which did not possess either the H or the 10043 group antigen, only one showed competence. The results indicate that twitching mobility is not a prerequisite for competence.

Key words: *Streptococcus sanguis* transformation; competence twitching fimbriation.

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The isolation and study of strains of *Streptococcus sanguis* characterized by production of spreading zones around colonies on agar media, by twitching motility and by polar fimbriation was reported in preceding papers (7 8) Most of the strains belonged to sero-group H but a few strains belonged to a new sero-group, provisionally designated the 10043 group.

In the *Neisseriaceae* an association between twitching motility polar fimbriation and competence in transformation has been demonstrated (1 2, 5 17) The purpose of the present study was to test the spreading streptococci for competence in transformation, and to study the relation, if any between twitching and competence

MATERIAL AND METHODS

The strains are the same as those used in the preceding studies and include 8 reference strains and 101 strains isolated by us, 85 strains of spreading streptococci, 80 of group H and 5 of group 10043 and 16 strai of non-spreading alpha haemolytic streptococci which did not possess either the H or the 10043 group antigen.

Selection of St ptomycin distant Mutants

Eighteen h cultures in Todd Hewitt (TH) broth were centrifuged and the sediments used to inoculate 5 per cent human blood agar plates containing 1000 µg streptomycin per ml. The plates

incubated in a humid atmosphere at 35 C. Colonies appearing within 3–5 days were transferred to new blood agar plates with and without streptomycin to check that they were streptomycin-resistant and that the capacity for twitching was preserved. They were also sero-grouped to verify the presence of the correct group antigen.

Enzyme Extract

Streptomyces griseus NCTC 7807 was grown in the medium described by *McCarthy* (10) The enzyme was extracted as described by *Slade & Slamp* (16) and the activity measured by following the reduction of optical density (OD) of streptococcal suspensions at 600 nm in relation to a control, adjusted to OD 0.50

Production of DNA

The streptomycin-resistant mutants are grown in TH for 18 h (14) After addition of citrate at the termination of the growth period to prevent DNAse activity the bacteria are centrifuged down. To a 4 per cent (v/v) suspension of wet cells in saline, sufficient enzyme is added to give 95–100 per cent lysis in the course of 4 h at 37 C. DNA is precipitated by ethanol deproteinized and precipitated once more, and finally dissolved in 0.85 per cent saline. The content of DNA is determined by Dische's reaction. As a standard, 2-deoxyribose is used. The DNA solution is divided into suitable portions and stored frozen until use.

Transformation Methods

Preliminary experiments using varying incubation times and inoculum sizes were carried out using the strain "Challis-Pakula as donor of DNA and as recipient. Two different methods were decided upon:

No. 1 From an 18 h culture in TH 0 2 ml is transferred to a tube with 5 ml TH, which is incubated for 3 h and cooled down to 2–25 C. 0 1 ml samples are transferred to three tubes A, containing 1.9 ml TH with 24 μg DNA, B, containing 1.9 ml TH, and C, containing 1 9 ml TH with 24 μg DNA and 50 μg DNAse. The tubes are incubated in a water bath at 37 C for 15 min, when 50 μg DNAse is added to tube A. The tubes are left in the water bath for another 2 h Ten-fold dilutions are made in TH and 0 1 ml volumes of suitable dilutions are spread on blood agar plates with 100 μg streptomycin per ml of medium (tubes A and C) for selection of transformants or mutants, or without streptomycin (tube B) for colony counts.

The results are read after 48 h in humid atmosphere at 35 C. Transformants are transferred to fresh blood agar plates with 1000 μg/ml) and without streptomycin to check for streptomycin-resistance and surface spreading. They were also checked for possession of the group antigens.

No. 2 Since method No. 1 did not appear to be sufficiently sensitive as a screening method for competence, an adaptation of a method described by *Jyssum & Lie* (9) was also used: Blood agar plates containing approximately 20 ml of medium are covered with a solution of DNA containing 120 μg DNA, i.e. 6 μg/ml. The plates are left at room temperature for 1 h and in the refrigerator until the following day From 19 h cultures in TH 10 μl volumes are spread on quadrants of the DNA plates. The plates are incubated at 37 C for 4 h, after which the whole agar layer is lifted over to another plate with 10 ml blood agar containing 300 μg streptomycin/ml, i.e. 100 μg/ml final concentration after diffusion. Incubation in a humid atmosphere at 35 C for 48 h. Blood agar without DNA, transferred to streptomycin agar and blood agar without streptomycin are used as controls. This method turned out to be more sensitive than method No 1 and revealed competence in additional strains.

In addition to DNA from "Challis-Pakula DNA from streptomycin-resistant mutants of strain 15843 group H, (apparently incompetent) and two strains of group 10043 strains 10013 (competent) and 10281 (apparently incompetent) were also prepared. The latter DNAs were used to transform all strains appearing to be incompetent with "Challis-Pakula DNA as well as 14 strains which had given positive results.

RESULTS

Transformation method No 1 Seventy four strains were screened for competence. Twenty-six out of 69 group H strains and 3 out of 5 group 10043 strains were transformed by "Challis-Pakula" DNA. The strains "Challis-Pakula and NCTC 7868 "Challis" both showed high competence with numerous transformants, whereas NCTC 9124 "Wicky" as expected did not show any transformants. The numbers of transformants of the other competent strains varied from a few up to a number nearly as high as that in the case of the "Challis" strains.

Transformation method No 2 Among 95 strains which were tested with "Challis-Pakula" DNA, 72 strains, 68 of group H and 4 of group 10043 were competent, with numbers of transformants varying from a few up to numbers nearly as high as those in the case of the Challis" strains. Twenty-two strains 20 of group H 1 of group 10043 and

ATCC 10557 "Washburn Type II" gave negative results.

The strains which failed to be transformed by this DNA, and 14 strains which appeared to be competent, were tested with the 3 other DNAs, from 13843 10043 and 10281 Using DNA from 13843 only one additional strain strain 13843 itself was transformed. DNA from 10281 did not transform any strain which was not transformed by the "Challis" DNA. The 14 strains which were transformed by "Challis" DNA were also transformed by the three other DNAs.

Among the 16 non spreading strains which did not possess either the H or the 10043 group antigen, only one strain, 12291 was transformed.

DISCUSSION

The majority of the spreading strains, 66 out of 85 showed competence in tests using DNA from "Challis-Pakula" In tests using 3 other DNAs, one additional strain was found to be competent, and only if autologous DNA were used.

On the other hand, 14 competent strains which were tested by all 4 DNAs were transformed by all of them. Thus the competence of the different strains is seen to differ some being transformed by all DNAs tested, and one being transformed only by autologous DNA. Since all these strains were spreading and, most probably fimbriated, the differences can hardly be due to differences in fimbriation. If other DNAs had been tested, it seems probable that other strains also might have shown competence. Since the methods are essentially qualitative it would be premature to draw any conclusions from the variations in numbers of transformants in the competent strains. Conditions may not necessarily have been optimal for production of the maximal number of transformants in each case

In contrast to the results obtained with the spreading strains, only one out of 16 non spreading strains of α haemolytic streptococci was transformed by the DNA from "Challis-Pakula" This discrepancy might be presumed to be related to the absence or presence of twitching and, presumably of fimbriae, but it might equally well be explained as due to a relationship of the non-spreading strains to the spreading strains, less close than that be tween the latter strains. The non-spreading strains have not been examined as thoroughly as the spreading strains and have not been accurately classified.

The results obtained with the reference strains, however indicate that differences with respect to twitching cannot be the expla nation of differences in competence. Among the strains of group H two, NCTC 7863 and ATCC 10556 (both "Washburn Type I" (18)) produced spreading zones. Both strains were competent. But 5 strains, NCTC 7868 "Challis" "Challis-Pakula" NCTC 9124 "Wicky" NCTC 7865 and ATCC 19558 (both "Washburn Type I/II" (18)) have never produced spreading colonies in spite of close observation for a long time. Three of these strains, the two "Challis" strains and one of the two Type I/II strains (NCTC 7865) were competent. It is of particular significance that the "Challis" strain has long been recognized as a highly competent strain which has been extensively used as recipient in transformation studies.

Thus, our results indicate that twitching is not a prerequisite for competence in this species, as it has been shown to be in some *Moraxella* and *Neisseria* species (1 2, 5 17)

The apparent lack of association between twitching and competence in some sthains of *S sanguis* is not surprising in view of the data published about the production of a soluble competence factor in cultures of the "Challis" strain (3 4 11 12 13 15) Further studies seem to be needed if the mechanism of com petence in streptococci is to be completely clarified.

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HAEMAGGLUTINATION OF TWITCHING *STREPTOCOCCUS SANGUIS*

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Among 66, mostly twitching, polarly fimbriated strains of *Streptococcus sanguis* 35 agglutinated guinea pig erythrocytes (GPE) after cultivation in Todd-Hewitt broth (TH) and 21 strains agglutinated GPE only after growth in TH with 10 per cent horse serum (THS) Two of the positive strains were non-twitching and unfimbriated. Ten strains failed to haemagglutinate. Among 5 twitching strains belonging to the 10043 group, 3 agglutinated GPE after growth in TH and 2 only after growth in THS Among 35 non-twitching strains of α-haemolytic streptococci, only 6 agglutinated GPE after growth in TH, and among 8 negative strains which were tested after growth in THS, only 1 agglutinated GPE. Tests using 6 different kinds of erythrocytes (guinea pig, rabbit, sheep, horse, chicken, human) revealed that differences between these were slight only The results do not indicate that there is an absolute association between twitching and haemagglutination in *S sanguis*. The haemagglutination of *S sanguis* was not mannose-sensitive.

Key words *Streptococcus sanguis* twitching motility polar fimbriae; haemagglutination.

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The isolation of twitching, polarly fimbriated strains of *S sanguis* from the human pharynx was reported in previous papers (10 11) Studies of the competence in transformation of the strains (9) showed that the majority of strains were competent, but that twitching could not be an absolute requirement for competence because a few non-twitching strains showed competence.

The present paper reports studies of the capacity of the strains for haemagglutination, and the correlation of twitching and haemagglutination.

MATERIAL AND METHODS

The strains are the same as those studied before (9 10 11) The methods for haemagglutination and mannose sensitivity tests were as described by *Duguid et al.* (4) with minor modifications. The strains were grown in TH at 35 C for 20 h. One tenth of a ml from these cultures was transferred to fresh tubes with 10 ml TH and grown at 35 C for 45 h, when the cultures were centrifuged, the supernatant decanted, and th tubes drained for 5 The packed bacteria were resuspended in the small volume of fluid remaining in the t bes, and the suspensions were used for haemaggl tination as described (4)

In some experiments the final culture was made in THS or in THS containing 1 per cent yeast extract (THSY) in a attempt to stimulate the dev lopment of fimbriae.

GPE (3 per cent packed cells, v/v) were used,

TABLE 1 *Agglutination of Different Kinds of Erythrocytes by Streptococcus sanguis*

Strain	Medium	Result of preceding test with guinea pig cells	Kind of erythrocytes					
			Guinea pig	Rabbit	Sheep	Horse	Chicken	Human
"Challis-Pakula"	TH	–	–	–	–	–	–	–
	THS		–	–	–	–	–	–
10115a	TH	+	–	–	–	–	(+)	(+)
	THS		++	++	++	++	++	++
7a	TH	–	–	–	–	–	–	–
	THS		(+)	–	–	–	(+)	(+)
14567	TH	–	–	–	–	–	–	–
	THS	(+)	–	–	(+)	(+)	+	–
14496	TH	+	+	+	+	+	+	+
	THS		+	+	+	+	+	+
2974	TH	+	+	+	+	+	+	+
	THS	+	+	+	+	+	+	+
NCTC 7863	TH	–	–	–	–	–	–	–
	THS	–	–	–	–	–	–	–
NCTC 7865	TH	–	–	–	–	–	–	–
	THS	–	–	–	–	–	–	–
10227	TH	+	(+)	–	–	–	–	–
	THS		–	–	–	–		–
12318	TH	+	–	–	–	–		–
	THS		+	+	+	(+)	(+)	(+)
ATCC 10556	TH	–	–	–	–	–	–	–
	THS	+	+	+	+	(+)	+	–
10659	TH	–	–	–	–	–	–	–
	THS		–	–	–	–		–

TH Todd-Hewitt broth, THS Todd Hewitt broth with 10 per cent horse serum. + rapid, coarse agglutination. (+) finely granular and/or slow agglutination. – no agglutination.

but in a few experiments erythrocytes from rabbits, sheep, horse chicken and human were used for comparison. Cells for these experiments were grown in 100 ml portions of TH or THS.

RESULTS

Among 86 strains of group H, TH-cultures of 55 agglutinated GPE in the first attempt. In 36 cases, the agglutinates were coarse and appeared within 30 s. In 19 cases, the agglutinates appeared more slowly (>30 s to several min) and/or were finely granular and difficult to see. Thirty-one strains gave negative results if TH-grown cells were used but 21 of these strains agglutinated GPE after growth in THS. THSY-grown cells did not give better results. Twelve of the 21 strains gave coarse and 9 gave fine agglutinates. THS itself did not agglutinate GPE.

As regards the reference strains "Challis-Wicky" (both non-twitching) NCTC 7863 and ATCC 10556 (derived from the same strain and both twitching) haemagglutinated, whereas "Challis-Pakula" NCTC 7865 and ATCC 10558 (both non-twitching derived from the same strain) failed to haemagglutinate.

Among 5 twitching strains of group 10045

(11) 3 produced coarse agglutinates after growth in TH, and 2 agglutinated GPE, one with coarse and one with fine agglutinates, after growth in THS.

Among 35 TH-cultures of non-twitching α-haemolytic streptococci only 6 agglutinated GPE, 3 with coarse and 3 with fine agglutinates. Eight of the 29 negative strains were tested after growth in THS, and only 1 agglutinated GPE.

A number of strains which had given strong haemagglutination were tested for mannose-sensitivity with negative results.

Twelve strains, 5 of which had given positive results after growth in TH, 2 only after growth in THS, and 5 negative in both media were grown in 100 ml of TH and THS and tested against the 6 kinds of erythrocytes specified in the preceding section. The results are shown in Table 1. In most cases it did not seem to make any difference whether one or the other type of erythrocyte were used. In one case (10115a) the TH culture was weakly positive with chicken and human cells only. In a second case (7a) the THS culture caused weak agglutination of GPE and strong agglutination of chicken and human cells, but not of the other types. In a third case (14567) the THS culture agglutinated sheep and horse cells weakly and chicken cells more strongly but none of the other types, and in a fourth case (10227a) the TH culture produced a questionable agglutination of GPE only.

DISCUSSION

Duguid & *Gillies* (5) and *Duguid et al.* (4) showed that fimbriae (apparently mainly peritrichously distributed) were common in many species of *Enterobacteriaceae*. They occurred with two types, 1 and 2. The presence of fimbriae of type 1 but not of type 2, was associated with haemagglutinating capacity and adhesiveness. The haemagglutination was mannose-sensitive.

In *Moraxella* and *Neisseria* species fimbriae of polar location where this could be determined have been demonstrated. These fimbriae have been shown to be associated with twitching motility competence in transformation, adhesiveness and, in some cases, with virulence (1 2 3 6 7 8, 12 13 14 15 16 17).

The twitching strains of *S. sanguis* have fimbriae of the polar kind, and it was of interest to find out whether these fimbriae also are associated with other biological traits. In the previous study (9) it was found that competence in transformation was very common in twitching strains, but that a few of the old "classical" strains also were competent although they could not be shown to twitch or to possess fimbriae.

The results obtained in this study indicate that capacity for haemagglutination is very common in the twitching strains of *S. sanguis* apparently more common than in other α-streptococci, but the association of these characters is not absolute. Thus the classical group H strains "Challis" and "Wicky" agglutinated GPE, although they have never shown signs of twitching and have been examined for fimbriae with negative results (10). If these strains actually are incapable of producing fimbriae, these appendages can at least not be the only basis for haemagglutination in this species. Indeed, there is no proof that they have anything to do with haemagglutination. The fact that some of the non-twitching α haemolytic streptococci were found to agglutinate GPE is suggestive, but of unknown significance.

The results of the tests appeared to be capricious with variation between tests. If fimbriae are involved, this might possibly depend upon differences in the degree of fimbriation under different cultural conditions, or upon damage to the fimbriae by the handling of the culture (centrifugation resuspension of the cells etc.). Negative results of such tests may therefore be misleading. Thus, our results do not demonstrate that there is an association between fimbriation and capacity for haemagglutination. More extensive studies would be necessary to clarify the basis for haemagglutination in these organisms.

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IDENTIFICATION OF PARAMYXOVIRUS-SPECIFIC HAEMOLYSIS-INHIBITING ANTIBODIES SEPARATE FROM HAEMAGGLUTINATING-INHIBITING AND NEURAMINIDASE INHIBITING ANTIBODIES

I Sendai Virus Haemolysis-inhibiting Antibodies

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Örvell, C. Identification of paramyxovirus-specific haemolysis-inhibiting antibodies separate from haemagglutinating-inhibiting and neuraminidase-inhibiting antibodies. I Sendai virus haemolysis-inhibiting antibodies. Acta path. microbiol. scand. Sect B 84 441–450, 1976.

Egg-grown Sendai virus was used for preparation of rabbit hyperimmune sera directed against purified whole virus and pronasetreated projectionless virus particles. These sera and convalescent sera after natural Sendai infection in guinea pigs were studied in haemolysis-inhibition (HLI), haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests both before and after absorption with Tween 80-ether (TE) treated virus preparations. In addition, neutralization tests using the different sera were carried out. HI and NI antibodies and the major population of neutralizing antibodies in convalescent sera were removed by absorption with TE treated virus material without changing the titre of non-HI HLI antibodies. Rabbit hyperimmune sera directed against projectionless virus particles exhibited HLI antibody titres in marked excess of HI and NI antibody titres, whereas this was not found in sera against purified whole virus. In contrast, absorption of sera against projectionless particles eliminated HI antibodies without changing the titre of non-HI HLI antibodies. The protein composition of antigenic preparations used in absorption experiments and for preparation of sera was investigated by SDS-polyacrylamide-gel electrophoresis. TE treatment had no significant effect on the polypeptide pattern of Sendai virus. Pronase-treatment predominantly affected the two glycosylated proteins of Sendai virus. The larger glycoprotein was not detectable in pronasetreated projectionless virus particles, whereas the smaller glycoprotein was present in reduced quantities.

Key words: Paramyxovirus-specific antibodies; haemolysis-inhibition; haemagglutination-inhibition; neuraminidase-inhibition.

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Three different biological activities are associated with the envelope of paramyxoviruses, haemolysis (HL), haemagglutinating (HA) and neuraminidase (NA) activity. It has been known for a long time that these biological activities are differently affected by various chemical treatments. For example

the HL activity of Sendai virus is relatively more sensitive than other activities to the action of trypsin and diisopropylfluorophosphate (15 16) Treatment of paramyxoviruses with ether destroys the HL activity but not HA and NA activities (33 34)

During recent years, data concerning the relationship between biological activity and the protein structure of the envelope of paramyxoviruses have started to accumulate. SV 5 NDV and Sendai virus have both haemagglutinating and neuraminidase activity located on the same glycoprotein (9 24 25 26 28, 31 35) A smaller glycoprotein has also been identified in paramyxoviruses (6 11) On the basis of studies of Sendai virus and SV 5 it has been proposed that the smaller glycoprotein is involved in haemolysis, cell fusion and the penetration of virus into cells (7 9 26 27)

Only few immunological studies of antibodies directed against various biological activities of the envelope have been carried out. By serological tests, *Seto et al.* (32) characterized specific antibodies directed against the two glycoprotein structures of NDV There are no reports on similar studies of Sendai virus. In human measles sera, a preferential haemolysis-inhibition (HLI) over haemagglutinating-inhibiting (HI) antibody response was encountered in about ten per cent of the ..ult population (23) and later a technique ..as been developed for a separate identification in all sera of antibodies giving HLI but not HI against measles virus (20)

A separate occurrence of HLI antibodies was also observed in rabbit hyperimmune sera against measles virus particles from which the major part of surface projection had been removed by treatment with trypsin (20)

The aim of the present study was to make a separate identification of HLI antibodies from HI and NI antibodies in antisera against Sendai virus. Attempts were also made to determine the correlation of neutralizing antibody activities to antibodies against the different envelope components.

MATERIALS AND METHODS

Virus. Sendai virus was obtained from Dr *K. Cantell* the National Serum Institute, Helsinki, Finland. The virus was grown in the allantoic sac of 11 or 12 days old embryonated chicken eggs. The material was harvested after incubation for 3 days at 37 C. *Isotope labelling* Eggs infected with Sendai virus were labelled 16 hours after infection by injection into the allantoic sac of ^{3}H amino acids from Chlorella protein hydrolysate for labelling of viral proteins or ^{3}H glucosamine (specific activity$>$10,000 mCi/mmol.) for labelling of glycoproteins, 20 and 40 μCi per egg, respectively

Both isotopes were obtained from the Radiochemical Centre, Amersham.

Preparation of purified virions and projection-free virus particles. Purified virions were prepared from egg-grown material The material was clarified at 200 × g for 10 minutes and then centrifuged at 20,000 rev/min for 60 minutes in R21 or SW25 II rotors, Spinco, Beckman Instruments.

The virus-containing pellet was suspended in phosphate buffered saline (PBS) and layered on top of discontinuous sucrose gradients, composed of 5 ml 50 per cent (w/w) and 15 ml 25 per cent sucrose solutions in PBS and centrifuged at 18,000 rev/min for 90 minutes in a SW25 I rotor Virus particles banding at the interphase between the sucrose layers were used for immunization and for preparation of projectionless particles Virions were treated with pronase (3 17 30) from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, at a final concentration of 0.5 mg/ml (specific activity 45 000 PUK units/g) at 37 C for 1 to 4 hours after dialysis against PBS. Pronase treatment under these conditions led to destruction of HA, HL and NA activities.

The effect of the treatment was monitored by HA tests. When HA activity no longer was detectable, the material was layered on top of a discontinuous gradient composed of 2 ml 65 per cent, 4 ml 50 per cent and 15 ml 25 per cent sucrose solutions and centrifuged at 18,000 rev/min for 90 minutes in a SW 25 I rotor The visible band was identified as projectionless particles by complement fixation (CF) tests using convalescent sera and by electron microscopy and used for immunization.

SDS polyacrylamid -gel electrophoresis. The procedure essentially followed the method of *Maizel* (1) Prior to electrophoresis radioactive samples were dissolved in 1 per cent SDS and 1 per cent 2-mercapto-ethanol and treated at 100° C for 2 minutes, after which 10 per cent glycerol was added. Electrophoresis was performed in 10 per cent polyacrylamide gels (0.20 per cent NN'-methylenbisacrylamide) 100 mm length and 5 mm diameter containing 0 1 per cent SDS. 3 per cent polyacrylamide was used as spacer gel. Electro-

phoresis was carried out at 2mA per gel for 6 hours with bromphenol blue as a marker dye. At the end of the run, gels were removed from the tubes, frozen and sliced in equally long pieces. Each gel piece was dissolved in 0.5 ml hydrogen peroxide overnight at 37° C. The radioactivity was determined after addition of 5 ml Aquasol to each sample.

Sera. Rabbit hyperimmune sera were produced by injection of 4 ml of antigen mixed with Freund's complete adjuvant intramuscularly followed by an intravenous booster 3 weeks later (20). Serum samples were collected at different time intervals and the animals were exsanguinated one week after the booster.

In addition, convalescent sera were prepared. Mice and guinea pigs were infected via the respiratory route with Sendai virus. Three weeks later the animals were either exsanguinated or given an intravenous booster of 0.1 to 0.5 ml of purified virions. The latter group of animals were exsanguinated after another 5 days.

Absorption of sera. TE treated (10, 18) Sendai virus materials of varying concentrations were mixed with sera. After incubation at room temperature for 1 hour and overnight at +4 C, the mixtures were centrifuged at 35,000 rev/min for 2 hours in R40. The supernatants were collected and tested. Excess HA was removed by absorption with chicken erythrocytes.

Serological tests. Egg-grown material was used as antigen in HI, HLI and NI tests.

HI tests. These tests were performed in microplates as previously described (19). 4 HA units of TE treated (10, 18) virus antigen were used in the tests. Chick erythrocytes from one-day-old chickens were used and allowed to settle for 45 minutes at +4 C.

HLI tests. The technique used has previously been described in detail (19). Human O blood cells (more stable against spontaneous haemolysis than chick erythrocytes) were chosen for HLI tests. In addition, inactivated calf serum at a final concentration of 0.5 per cent was added to increase the stability of erythrocytes.

NI tests. These tests were performed according to *Panker* (22) with the exception that 0.05 M [illegible] buffer pH 5.1 was used (2). 0.1 ml of freshly frozen foetal calf serum was used as source of fetuin in the tests. N-acetyl neuraminic acid was determined according to the procedure of [illegible] (36) except that the colour was extracted into n-butanol containing 5 per cent (V/V) [illegible] hydrochloric acid (1). The antigen [illegible] NI test were diluted to give an OD [illegible] of 0.4 to 0.7 per 0.05 ml of [illegible] (1) [illegible] extinction of free N-acetyl [illegible] acid in foetal calf serum amounted to [illegible] to 0.12.

Neutralization (NT) and neutralization-enhancement (NE) tests. Tests with Sendai virus in the absence (NT) and presence of anti-gamma globulin, referred to as neutralization-enhancement (NE) tests (5, 13, 19, 21) were performed in the allantoic sac of 11 or 12 days old embryonated chicken eggs. Antisera against rabbit and guinea pig gamma globulins used in NE tests were provided by the Department of Immunology, National Bacteriological Laboratories, Stockholm. Equal volumes (0.20 ml) of serial twofold dilutions of serum and virus material containing 100-200 EID_{50} (50 per cent egg infectious doses) per 0.1 ml were mixed and incubated for 1 hour at room temperature and at +4 C overnight. In NE tests the corresponding dose of virus was added per serum dilution, but in a volume of 0.13 ml. After incubation of the samples for 1 hour at room temperature and overnight at +4 C 0.07 ml of anti-rabbit or anti-guinea pig gamma globulin diluted 1/4 was added per antigen-antibody mixture and the incubation was continued for a further 2 hours at room temperature. 0.15 ml of the final mixture was inoculated into the allantoic sac of 2 eggs per dilution. The eggs were incubated for 3 days at 37 C. Eggs devoid of detectable HA at a dilution of 1:4 were considered uninfected. The 50 per cent neutralization titres of sera were calculated.

RESULTS

Characterization of Projectionless Particles Used for Immunization

Purified virus particles labelled with ³H glucosamine or ³H amino acids were treated with pronase at a final concentration of [illegible] per ml until all HA activity had disappeared and was then centrifuged on a [illegible] sucrose gradient (see Materials and Methods). The distribution of [illegible] of one pronase treated and [illegible] control virus preparation after [illegible] ous sucrose gradient centrifugation [illegible] Fig. 1.

In the pronase treated [illegible] the major part of the [illegible] not penetrate the 25 per [illegible] whereas particles [illegible] cosamine label and [illegible] between 50 and 25 [illegible] electron micros[illegible] appeared to repr[illegible] major part of [illegible] moved.

pronase treated virus preparations, but the protein was clearly discernible.

In Fig 3 C the glycosylated proteins of Sendai virus, VP2 and VP4 are shown after labelling with ³H glucosamine. In addition, glucosamine label was also detected near the front of the gel. This peak was not resolved in our gels, but many represent the small glycoprotein (SGP) described by other workers (7 28) After glucosamine labelling, neither VP2 nor VP4 could be detected in the pronase-treated virus preparations (see Fig. 3 D) All glucosamine labelled material was detected near the front of the gel.

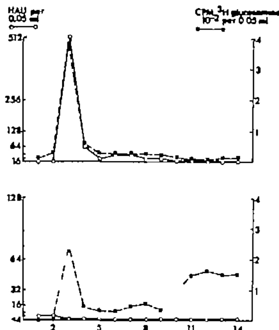


Fig 1 Distribution of glucosamine counts of pronase-treated haemagglutinatinin free Sendai virus (lower part) and the corresponding untreated preparation (upper part) after centrifugation in a discontinuous sucrose gradient (2 ml 65 per cent, 4 ml 50 per cent and 15 ml 15 per cent sucrose) at 18,000 rev/min for 90 minutes in a Spinco SW 25 rotor The amount of pronase-treated material applied to the gradient was two times that of the control material. The following activities were recorded haemagglutination (○) and ³H glucosamine counts (■)

The proteins and glycoproteins of untreated and pronase-treated Sendai virus particles sedimenting to the interphase between 50 and 25 per cent sucrose after discontinuous gradient centrifugation were investigated by SDS-polyacrylamide-gel electrophoresis (Fig 3) The designation of the major viral polypeptides is according to the designation used by *Shimizu et al* (28) The polypeptide on which HA and NA activity is located, VP2 (26 27 28) was not detectable in the pronase treated virus preparations (Fig 3 B) The haemolytic activity of Sendai virus has been reported to be associated with VP4 (7 26) A relative depression of VP4 in relation to VP3 (the nucleocapsid protein) and VP5 (the membrane protein) was observed in the

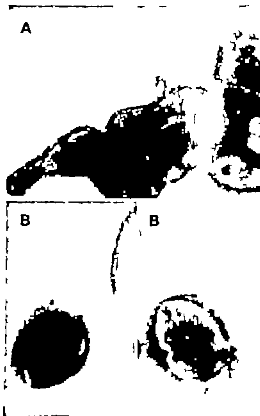


Fig 2 Morphology of intact (A) and pronase-treated (B) Sendai virus particles. Some remaining projections are seen on the pronase-treated particles. Specimens negatively stained with 1 per cent sodium tungstic silicate Magnification × 38,000 (A) × 48,500 (B)

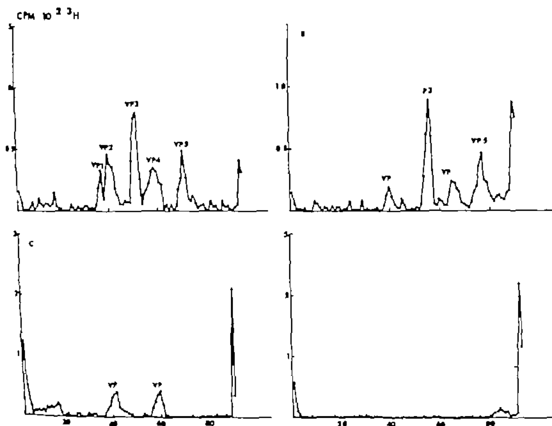


Fig. 3 SDS-polyacrylamide-gel electrophoresis of purified untreated and pronase-treated Sendai virus particles after centrifugation in a discontinuous sucrose gradient (2 ml 65 per cent, 4 ml 50 per cent and 15 ml 25 per cent sucrose) at 18,000 rev/min for 90 minutes in a Spinco SW 25 rotor
A: ³H amino acid labelled purified virus particles.
B ³H amino acid labelled pronase-treated particles.
C ³H glucosamine labelled purified virus particles.
D ³H glucosamine labelled pronase-treated particles.
The virions were dissolved in SDS and mercaptoethanol and run on 10 per cent polyacrylamide gels. Migration is from left to right. The designation of the polypeptides is made according to *Shimizu et al.* (28)

Effect of TE Treatment on Sendai Virus

The effect of TE treatment on purified Sendai virus was studied both by biological tests and by SDS-polyacrylamide-gel electrophoresis. The HL activity of the virus was destroyed by the treatment whereas HA activity was increased as expected (10, 18). When ³H-amino acid labelled virions were subjected to TE-treatment, 40 to 50 per cent of the label was recovered after the treatment. SDS-polyacrylamide-gel electrophoresis of TE treated preparations showed no profound effects on the polypeptide pattern of Sendai virus. All viral proteins were clearly discernible (Fig 4). No significant loss of VP4 in relation to VP2 was observed in five consecutive experiments. A relative loss of VP3 and VP5 in relation to VP2 and VP4 was observed in most experiments.

Demonstration of Antibodies Directed against Envelope Components of Sendai Virus

Sendai convalescent sera from mice and guinea pigs contained HLI and HI antibodies only in low titre and NI antibodies were barely demonstrable or absent. In orr

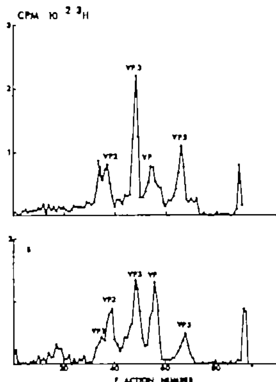


Fig 4 The polypeptide pattern of ^{3}H amino acid labelled purified virions before (A) and after (B) Tween 80-ether treatment.

to increase the antibody titres some animals were given an intravenous booster of purified virions. After the booster the animals showed a marked rise in antibody titres and in a few animals, the sera exhibited a marked excess of HLI to HI antibody titres. The latter sera were selected for absorption experiments.

a) Evidence for the Occurrence of HLI Antibodies Distinct from HI and NI Antibodies

Absorption of convalescent sera using TE treated antigen led to a reduction of HI and NI antibodies without significant reduction of HLI antibody titres (Table 1) In some sera, HI antibodies were removed more readily than NI antibodies.

Rabbit hyperimmune sera directed against projectionless particles contained HLI antibodies in marked excess of HI and NI antibodies compared to sera from rabbits immunized with purified virions (Table 2) Correlation between the titres of HI and NI antibodies was good in both types of sera. In absorption experiments using carefully titrated amounts of antigen, HI antibodies could be removed in sera against projectionless particles without significant reduction of HLI antibody titres (Table 2) When in-

TABLE 1 *D termination of Different Antibody Activities in Three Guinea Pig Convales ent Sera and T o use Convalescent Se a before and after Absorption with Tween 80-ether T eated Sendai Virus*

Serum	Absorption with TE treated Sendai virus	HI	HLI	NI	NT	NE
Guinea pig con alescent 1	0	2048	16,000	64	4096	64,000
	+	<32	16,000	≤16	1024	16,000
Guinea pig convalescent 2	0	1024	4096	32	1024	32,000
	+	<32	4096	≤ 8	256	8,000
Guinea pig convalescent 3	0	1024	4096	64	2048	–
	+	64	2048	32	512	
Mouse convalescent 1	0	64	128	≤ 4		
	+	16	128			
Mouse convalescent 2	0	64	128		–	
	+	<4	64			–

Guinea pigs received an intravenous booster with purified irions 3 weeks after exposure to irus (see Materials and Methods)

TABLE 2. *Antibody Response of F Rabbits after Hyperimmunization with P ojectionless or Purified whole Virus Particle of Sendai Virus*

Rabbit hyperimmune serum against	Absorption with TE treated Sendai virus	HI	HLI	NI	NT	NE
projectionless virus particles 1	0	32	512	4	<4	32,000
	+	<4	256	–	–	–
projectionless virus particles 2	0	128	1024	8	≤8	64,000
	+	32	512	8	–	–
projectionless virus particles 3	0	128	1024	16	64	128,000
whole virus 1 before booster	0	512	1024	32	128	64,000
	+	128	256	<16	–	–
	+	≤32	≤64	–	–	–
whole virus 2 after booster	0	2048	4096	128	2048	256,000
	+	256	1024	64	–	–

creasing amounts of antigen were used in the absorption experiments, a reduction of HLI antibody titres was observed in these sera. The behaviour of rabbit hyperimmune sera directed against purified virions was different from that of rabbit hyperimmune sera directed against projectionless particles in absorption experiments. In the former type of sera, a reduction of *both* HI and HLI antibodies was observed and it was not possible to obtain samples retaining HLI but not HI activity

b) Correlation of NT and NE Antibody Activities to Antibodies against Envelope Components

Removal of HI antibodies in convalescent sera by absorption with TE antigen resulted in an elimination of the major fraction of NT and NE antibodies (Table 1) However a certain proportion of NT and NE antibodies still *remained* after absorption.

The most striking difference between rabbit hyperimmune sera directed against projectionless particles and purified virions was observed in NT tests (Table 2) Two of the three sera directed against projectionless particles were devoid of detectable NT antibodies *although some HI* and a high titre of HLI antibody activity was detectable the third serum only exhibited low titres of NT antibodies. This difference was not observed after anti-gamma globulin had been added to the test. A pronounced increase in titres was observed and there was a good correlation between HLI and NE antibody titres in all sera studied.

DISCUSSION

Proteins as well as lipids are required if the biological activity of HL is to be expressed (6, 8, 9) The biological activity of the HL is destroyed by TE treatment, but it is not known whether this results in a destruction of the immunogenic properties of the HL. TE treatment did not selectively remove any polypeptide of Sendai virus. Since a reduction of HLI antibodies did not occur in convalescent sera after absorption, an alteration of the immunogenic properties of the haemolysin component(s) may have occurred. The same conclusion was previously drawn in studies of measles virus (20)

In accordance with recently published results on measles virus (20) the behaviour of rabbit hyperimmune sera against purified virions of Sendai virus differed from that of convalescent sera in absorption experiments using TE treated antigen. In the latter

of sera HLI antibodies were not removed by absorption in contrast to results obtained if rabbit hyperimmune sera were used. The reason why results obtained by the two kinds of sera used in absorption experiments differed, remains an object of speculation. An efficient replication of Sendai virus which is known to occur in its natural host may be a factor of importance for production of a strong non-HI HLI response.

It cannot be excluded that the HLI antibody response of rabbit hyperimmune sera to purified virions of Sendai virus obtained in the present study was comparatively weak, which may explain the failure to demonstrate HLI antibodies after HI antibodies had been removed from rabbit hyperimmune sera. In studies of measles virus (20) HLI antibodies were not either detectable in TE antigen absorbed rabbit hyperimmune sera against whole virus.

Various proteolytic enzymes have the capacity to remove the projections of paramyxoviruses (3 4 6 17 20) Pronase treated Sendai virus used in immunization studies were devoid of HL, HA and NA activity Hyperimmunization of rabbits using antigenic preparations of this type resulted in a comparatively strong HLI antibody response, whereas HI NI and NT antibody responses e weak or absent. This must be inter to mean that the HL remains in an immunogenic form after the treatment. The finding that VP4 (the glycoproteins associated with HL activity) is relatively less sensitive to the action of pronase than VP2 (the glycoprotein associated with HA NA activity) is in fair agreement with this view A similar selective sensitivity of the two glycoproteins to the action of fungal semi-alkali protease and to chymotrypsin has recently been shown by *Shimizu & Ishida* (29) In this investigation VP4 could not be detected in H glucosamine labelled pronase-treated virus particles. A selective removal of the carbohydrate part of this glycoprotein is apparently an additional effect to be exerted by pronase

The significance of the glucosamine label moving near the front of the gel remains unknown. The presence of a small glycoprotein (SGP) in Sendai virus has been described by some workers (7 28) but other workers have not found this glycoprotein (14 26)

In sera directed against projectionless particles, HI antibodies were removed without significantly affecting the titre of non-HI HLI antibodies. In this respect, the behaviour of hyperimmune sera directed against projectionless particles was similar to that of convalescent sera, but not to that of hyperimmune sera directed against purified virions. Similar findings were reported by *Norrby & Gollmar* (20) in studies of measles virus. However the results obtained in absorption experiments using convalescent and hyperimmune sera may not be comparable, as the two kinds of Sendai antisera are formed under different conditions of immunization. It is difficult to explain why a correspondingly efficient non HI HLI response was obtained when projectionless virus particles were used for immunization, but not when whole virus was used as immunogen. The different results to be obtained with the two kinds of rabbit hyperimmune sera in absorption experiments may also be due to the fact that HLI antibodies were present in marked excess of HI antibodies in sera against projectionless particles. Consequently relatively less TE treated antigen was required to remove HI antibodies in sera against projectionless particles. When larger amounts of antigen were used, a reduction of HLI antibody titres was observed also in these sera.

A reduction of both NT and NE antibody titres was observed in convalescent sera after HI antibodies had been removed by TE antigen absorption.

In rabbit hyperimmune sera, two sera directed against projectionless particles were devoid of NT antibodies in the presence of HI antibodies. It cannot be excluded that pronase-treatment may induce minor structural changes in antigenic determinants resulting in antigen-antibody bindings of low avidity which can be detected in HI but not in NT tests.

In sera against projectionless particles, rela

tively large increases in titres were observed after antigamma globulin had been added to the test.

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IDENTIFICATION OF PARAMYXOVIRUS-SPECIFIC HAEMOLYSIS-INHIBITING ANTIBODIES SEPARATE FROM HAEMAGGLUTINATING INHIBITING AND NEURAMINIDASE-INHIBITING ANTIBODIES

2. NDV and Mumps Virus Haemolysis-inhibiting Antibodies

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Örvell, C. Identification of paramyxovirus-specific haemolysis-inhibiting antibodies separate from haemagglutinating inhibiting and neuraminidase-inhibiting antibodies. Acta path. microbiol. scand. Sect. B, 84: 451–457. 1976.

Egg-grown Newcastle disease (NDV) and mumps virus were used for preparation of rabbit hyperimmune sera against purified whole virus and projectionless virus particles. These sera and convalescent sera after natural NDV and mumps infections in chickens and human subjects, respectively, were studied in haemolysis-inhibition (HLI), haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests both before and after absorption with Tween 80-ether (TE) treated virus preparations. In addition, neutralization tests using the different sera were carried out. HI and NI antibodies and the major population of neutralizing antibodies in convalescent sera were removed by absorption with TE treated virus material without changing the titre of non-HI HLI antibodies. Rabbit hyperimmune sera directed against projectionless virus particles exhibited HLI antibody titres in marked excess of HI and NI antibody titres, whereas this was not found in sera against whole virus. Absorption with TE treated virus material resulted in removal of all demonstrable antibody activities in sera against whole virus. The corresponding absorption of sera against projectionless particles eliminated HI antibodies without changing the titre of non-HI HLI antibodies. In rabbit hyperimmune sera, HI antibodies were of primary importance in neutralization tests. After addition of anti-gamma globulin to the test, an efficient neutralization was observed if mumps non-HI HLI antibodies were used whereas this was not found if NDV non-HI HLI antibodies were used.

Key words: Paramyxovirus-specific antibodies; haemolysis-inhibition; haemagglutination-inhibition; neuraminidase-inhibition.

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In the accompanying paper (21) the present knowledge concerning the relationship between biological activities and the protein structure of the envelope of paramyxoviruses was surveyed. As regards the three paramyxoviruses studied, SV5, NDV and Sendai virus, the haemagglutinating activity as well as the

neuraminidase activity are located on the same glycoprotein (4 12, 13 14 16, 17 20) A smaller glycoprotein of SV5 and Sendai virus has been proposed to be involved in haemolysis (3 4 14 15) Similar studies have not yet been carried out with mumps virus.

Only few immunological studies of antibodies directed against various biological activities of the envelope have been reported. *Seto et al* (18) isolated the two glycoprotein structures of NDV Immunization of rabbits with the smaller glycoprotein gave rise to HLI antibodies without HI NI and NT antibodies. Antibodies against the larger glycoprotein also interfered with cell fusion and haemolysis.

Non HI HLI antibodies were also identified in studies of measles virus (10) A selective removal of HI antibodies was achieved in human convalescent sera by absorption with TE treated material. In addition a separate occurrence of HLI antibodies was observed in rabbit hyperimmune sera against virus particles from which the major part of surface projections had been removed. In this investigation, techniques similar to those used in the latter trial were used in the study of other members of the paramyxovirus group. The aim of the present work was to identify HLI antibodies separate from HI and NI antibodies in antisera against NDV and mumps virus. It was also considered of importance to determine the correlation of NT and NE antibody activities to antibodies against the different envelope components.

MATERIALS AND METHODS

Virus and cell cultures. Newcastle disease virus (Montana strain) was kindly provided by Dr *M Lagercrantz* the National Bacteriological Laboratories, Stockholm, and mumps virus was derived from an isolate made at the National Bacteriological Laboratories. NDV and mumps virus were prepared in the allantoic sac of 8 to 12 days old embryonated chicken eggs. The materials were harvested after incubation for 3 and 7 days at 37° C, respectively. The viruses were also propagated in a green-monkey kidney cell line (Vero) free from detectable contamination by mycoplasma and used as antigen in neutralization tests.

Preparation of purified virions and projectionless virus particles. The techniques used for preparation of purified virions and projectionless virus particles were described in the accompanying paper (21) For preparation of projectionless virus particles, NDV and mumps virus were treated with pronase at a final concentration of 0.5 and 0.25 mg per ml respectively at 37 C for from 1 to 4 hours. The effect of the treatment was monitored by HA tests. When the HA activity no longer was detectable, the projectionless particles were centrifuged in a discontinuous sucrose gradient to the interphase between 50 and 25 per cent sucrose (21)

Sera. Rabbit hyperimmune sera were prepared in the same manner as Sendai virus and previously described (21) Chicken convalescent sera against NDV were prepared by intranasal administration of 0 1 ml doses of different concentrations of virus, up to 10^4 infectious units per 0.1 ml. Serum samples from the animals were collected until day 39 postinfection.

Human convalescent sera collected within 2 months after onset of symptoms of regular mumps infection were obtained from the Virus Department, National Bacteriological Laboratories, Stockholm. Batches of human gamma globulin were obtained from A.B. Kabi, Stockholm, Sweden.

The procedure used for the absorption of sera with TE treated virus materials is the same as that applied to Sendai virus and previously described (21)

Serological tests. Egg-grown material was used as antigen in HI HLI and NI tests. The methods for HI HLI and NI tests followed essentially the techniques used in studies of Sendai virus (21) TE treated (5, 7) virus antigen was used in HI tests if not otherwise stated Erythrocytes from one-day-old chicks were used in HI tests with NDV virus, whereas monkey erythrocytes were used in tests with mumps virus Monkey erythrocytes were allowed to settle for 60 minutes at +37 C. In HLI tests, human O blood cells and monkey erythrocytes were used in tests with NDV and mumps virus, respectively NI tests were performed at pH 5.2 and 5.5 using untreated NDV and mumps virus, respectively (1)

Neutralization (NT) and neutralization-enhancement (NE) tests Tests with NDV and mumps virus in the absence (NT) and presence of anti-gamma globulin, referred to as neutralization-enhancement (NE) test (2, 6, 8 11) were performed in Vero cells as in the case of measles virus previously described (8) Antisera against rabbit chicken- and human gamma globulins, used in the NE tests were provided by the Department of Immunology National Bacteriological Laboratories Stockholm All anti-gamma globulin sera were used at a dilution of 1 4 Final readings of tubes were made after tests both with NDV and mumps virus had lasted for 7 days.

TABLE 1 *Antibody Response in Six Chickens Exsanguinated at Different Intervals of Time after NDV Infection*

Animal No.	Days after infection	Absorption with TE treated NDV	HI with untreated antigen	HLI	NI	NT	NE
1	10	0	256	1024	–	<4	256
2	14	0	512	1024	64	16	256
3	16	0	256	1024	64	8	256
4	27	0	512	2048	128	256	2048
5	28	0	512	1024	64	256	2048
		+	128	1024	64	64	256
		+	64	512	32	32	128
6	39	0	256	1024	64	256	–
		+	≤8	512	≤8	32	–

TABLE 2. *Determination of Different Antibody Activities in Five Rabbits Hyperimmunized with Projectionless or Nonsoluble whole Virus of NDV*

Rabbit hyperimmune serum against	Absorption with TE treated NDV	HI	HLI	NI	NT	NE
projectionless virus particles 1	0	≤4	256	<4	8	8
projectionless virus particles 2	0	64	512	8	16	16
	+	≤8	256	<4	8	8
whole virus 1 before booster	0	256	512	16	64	1024
	+	≤32	≤64	–	–	–
whole virus 2 before booster	0	512	1024	32	128	2048
	+	128	256	16	–	–
whole virus 3 after booster	0	4096	4096	128	2048	16 000

RESULTS

Demonstration of Antibodies Directed against Various NDV Envelope Components

Different antibody activities in chicken convalescent sera collected at various time intervals after infection were studied (Table 1).

HI antibodies were studied in tests including untreated antigen, since it, unexpectedly was found that these antibodies were not demonstrable if TE treated antigen was present in chicken convalescent sera. HI HLI and NI antibodies reached maximum titres after 10 to 15 days postinfection.

a) Identification of HLI Antibodies Separate from HI and NI Antibodies

In chicken convalescent sera, a significant reduction both of HI and NI antibodies without a reduction of HLI antibody titres could be accomplished by absorption with TE treated antigen (Table 1). In some sera, HI antibodies were removed more readily than NI antibodies.

In rabbit hyperimmune sera, HLI antibodies in the absence of HI and NI antibody activity was found in one of two sera directed against projectionless particles (Table 2).

The second serum directed against projectionless particles exhibited HLI antibodies in marked excess of HI and NI antibodies as compared with sera directed against purified whole virus (Table 2) Absorption of the second serum directed against projectionless particles resulted in the removal of HI and NI antibodies without a significant reduction of HLI antibody titres.

In rabbit hyperimmune sera directed against purified whole virus, removal of HI antibodies resulted in absorption experiments, in a concomitant reduction of titres of HLI antibodies (Table 2)

b) Correlation of NT and NE Antibody Activities to Antibodies against Envelope Components

In chicken convalescent sera, NT antibodies appeared later than HLI HI and NI antibodies (Table 1) Addition of anti-gamma globulin gave an increase in titres which was most pronounced in early convalescent sera with low titres of NT antibodies. Absorption of late chicken convalescent sera resulted in a reduction of neutralizing antibodies without significant reduction in the titres of HLI antibodies.

Although HLI antibodies were present, only weak NT antibody responses were encountered in rabbit hyperimmune sera directed against projectionless particles (Table 2) Addition of anti-gamma globulin resulted in a pronounced increase in titres in sera directed against untreated virus whereas no increase was observed in sera directed against projectionless particles.

Demonstration of Antibodies Directed against Different Components of Mumps Envelope

Convalescent sera after regular mumps infection and a few samples of human gamma globulin were studied in excess of the rabbit hyperimmune sera. In human sera, NI antibodies occurred in low titres or they were not detectable in most cases.

a) Identification of HLI Antibodies Separate from HI and NI Antibodies

A separate identification of HLI from HI antibodies was consistently achieved in absorption experiments with human mumps sera (Table 3)

In rabbit hyperimmune sera, the HLI antibody titres were in marked excess of HI and NI antibody titres in the sera directed against

T 3 *Determination of Different Antibody Activities in Mumps Convalescent Sera and Human Gamma Globulin before and after Absorption with TE Treated Mumps Virus*

Human mumps serum sample	Absorption with TE treated mumps virus	HI	HLI	NT	NE
Convalescent 1	0	64	128	16	64
	+	≤4	128	8	64
Convalescent 2	0	64	256	32	512
	+	≤16	256	16	256
Convalescent 3	0	128	1024	64	1024
	+	16	51	16	512
Convalescent 4	0	64	64		
	+	≤8	61		
Gamma globulin 1	0	1.8	256	32	256
	+	<16	128	≤8	128
Gamma globulin 2	0	1.8	128		
		<16	128		

TABLE 4 *Antibody Response in Three Rabbits after Hyperimmunization with Projectionless or Nonsoluble whole Mumps Virus*

Rabbit hyperimmune serum against	Absorption with TE treated mumps virus	HI	HLI	NI	NT	NE
projectionless virus particles 1	0	32	512	<4	32	2048
	+	≤4	512		32	2048
projectionless virus particles 2	0	32	512	<4	32	1024
whole virus 1 before booster	0	128	256	<4	32	512
	+	<16	≤64		–	–
whole virus 1 after booster	0	2048	2048	64	2048	16,000

TABLE 5 *Absence of Immunological Relationships between Paramyxovirus Haemolysins. HLI Tests Included Homologous and Heterologous Viruses on Absorbed Sera Free of Demonstrable HI or NI Antibodies*

Serum sample	Antigen	HLI		
		Sendai	NDV	Mumps
Guinea pig Sendai convalescent		2048	≤32	≤32
NDV projectionless particles		<16	256	<16
Mumps projectionless particles		<16	<16	512

projectionless particles (Table 4) In absorption experiments with rabbit hyperimmune sera directed against projectionless particles, HI antibodies could be removed without a reduction in HLI antibody titres.

In rabbit hyperimmune sera directed against purified whole virus, removal of HI antibodies resulted in a concomitant reduction of HLI antibody titres (Table 4)

b) Correlation of NT and NE Antibody Activities to Antibodies against Envelope Components

In absorption experiments using human mumps convalescent sera, only a two- or four fold reduction of NT antibodies was observed and there was no reduction in the titre of NE antibodies (Table 3) Only low titres of NT antibodies were observed in rabbit hyperimmune sera directed against projectionless particles (Table 4) Addition of anti-gamma globulin to the test gave more pronounced increases in titres in these sera than in sera directed against purified virions. Removal of HI antibodies did not change the titres of NT and NE antibodies in sera directed against projectionless particles.

Demonstration of the Specificity of Homologous HLI Antibodies

It was considered of interest to investigate whether there was an immunological relationship between the haemolysins of Sendai, NDV and mumps viruses. Absorbed sera containing HLI antibodies, but no HI or NI antibodies, were tested in HLI tests where the heterologous viruses were used. As seen in Table 5, no crossreactions were observed.

DISCUSSION

The results obtained in studies of NDV and mumps virus were essentially similar to the results obtained with Sendai virus which are described and discussed in the accompanying paper (21) It applies to all three viruses,

that non HI HLI antibodies separate from HI and NI antibodies could be identified both in convalescent sera and hyperimmune sera directed against projectionless virus particles after absorption with TE treated virus material. Similar results have previously been obtained in studies of measles virus (10).

With the exception of human mumps convalescent sera, a reduction both in NT and NE antibody titres was observed in convalescent sera after removal of HI antibodies by TE antigen absorption. The effect of the corresponding absorption of rabbit hyperimmune sera varied. In sera against whole virus, all antibody activities were removed whereas no change in NT and NE antibody titres occurred in sera against projectionless particles. However the latter sera contained only low titres of NT antibodies which in the case of mumps showed a pronounced increase in the titre upon addition of anti-gamma globulin. In this case HLI antibodies were probably responsible for NT and NE antibody activities.

In chicken convalescent sera NT antibodies appeared later than HLI HI and NI antibodies. The phenomenon that NT antibodies appear later than HI antibodies has been observed in immunization studies of chickens with β propiolactone-killed NDV and reported by *Sullivan et al* (19). Lower avidity of HI antibodies in early chicken convalescent sera and non-boostered rabbit *hyperimmune sera against whole virus may* also explain why ratios of NT to HI antibodies are lower in these sera.

In these sera and in sera directed against projectionless particles, relatively large increases in titres were observed after anti-gamma globulin had been added to the test. Only sera directed against projectionless particles of NDV formed an exception. Besides avidity of antibodies, the density and distribution of various antigenic components on the envelope can be expected to be a factor of importance in NT and NE tests. If haemolysin components are widely spaced on the envelope HLI antibodies may be unable to show activity in neutralization enhancement tests.

The significance of measles HLI antibodies *in vivo* in the protection against measles has been studied by *Norrby et al* (9). It was proposed that the failure of inactivated vaccines of measles virus might be due to the absence of envelope components capable of inducing non-HI HLI antibodies. High titres of NT antibodies are known to be present after immunization with preparations of this type. The conclusion to be drawn from these observations was that NT antibodies measured *in vitro* may not always represent an absolute indicator of the degree of *in vivo* neutralization. *The successful identification of HLI* antibodies against several other members of the paramyxovirus group will make it possible to extend studies of HLI antibodies and their significance in the protection against diseases caused by these viruses.

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BRIEF REPORTS

ISOLATION OF A PENICILLINASE PRODUCING STRAIN OF *NEISSERIA GONORRHOEAE*

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Ødegaard, K. & Solberg O. Isolation of a penicillinase producing strain of *Neisseria gonorrhoeae* Acta path. microbiol. scand. Sect. B 84 458-460, 1976.

A gram-negative diplococcus with the qualifications of a gonococcus was isolated from the urethra of a male infected with gonorrhoea in the Far East. The strain proved to be resistant to penicillin G and ampicillin as judged by the paper disc sensitivity test. The resistance was found to be caused by a powerful production of penicillinase which not only acted on penicillin G and ampicillin, but also on methicillin and partly on cephalosporin (cephalotin) by the test employed.

Key words *Neisseria gonorrhoeae* penicillin resistance; penicillinase production.

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Since 1959 the National Institute of Public [He]alth has tested isolated gonococcal strains for [sensi]tivity to penicillin G as a matter of routine — [ab]out 16,000 *isolates by the plate dilution method* and approximately 24,000 by the paper disc method. No strain has been found resistant to penicillin G. The least sensitive strains could only be characterized as "fairly sensitive" or having a "reduced sensitivity" to penicillin G. The minimal inhibitory concentration of the least sensitive gonococcal strains ever found in this laboratory by the plate dilution method was 2 μg/ml of penicillin G.

On July 29th, 1976 we were surprised to find a gonococcal isolate which seemed to be *completely* resistant both to penicillin G and to ampicillin as judged by the paper disc method. The present paper gives a short description of some qualities of this strain.

The strain—Gc 16634—was isolated from the urethra of a male patient infected in the Far East *a few days earlier and suffering from urethritis.* The sample arrived at the laboratory on a charcoal impregnated swab placed in solid Stuart medium and was streaked out on our selective "chocolate" agar medium containing colistin, lincomycin, nystatin and trimethoprim (3). After about 18 hours of incubation at 36 C in 5 per cent CO_2 atmosphere, oxydase-positive colonies of typical gonococcal appearance and consisting of gram-negative *diplococci were found. The diagnosis was further* based on fermentation of glucose but not of maltose and sucrose and no growth on nutrient agar at 22° C. The direct immunofluorescent test using anti-gonococcal globulin conjugated with fluorescein isothiocyanate (Difco) was also positive.

The sensitivity testing by the paper disc method (AB Biodisk, Stockholm) was performed on our "chocolate" agar medium without the inhibitors for contaminating growth (3) and showed no growth inhibiting zone around the penicillin G disc (10 μg/disc), the ampicillin disc (10 μg/ml disc) or the sulphonamide disc (sulpha-isodimidine 0.25 mg/disc) whereas the microbe showed full sensitivity to streptomycin, tetracycline and erythromycin. As we had never before seen a gonococcal strain where resistance to the penicillins was *of such degree the procedure was repeated twice* but the same result was obtained. We then extended the paper disc testing to other antibacterial agents with the following result: resistant to cotrimoxazol, methicillin, carbenicillin and colistin

slightly sensitive to kanamycin fairly sensitive to chloramphenicol, cephalosporin and gentamycin, and sensitive to nitrofurantoin and nalidixin. Testing for penicillinase production and sensitivity testing by the plate dilution method was then performed.

Method

The principle of penicillinase detection described earlier (4) was employed on the same chocolate agar medium as that used for the sensitivity testing by the paper disc method. Plates of 14 cm diameter were seeded by flooding, using a 3 ml suspension of a penicillin sensitive strain of *St ph. aureus* (P209) diluted so as to give a dense but not a confluent growth of colonies (1) Surplus inoculum was removed by a pipette and the plates were allowed to dry for about one hour t room temperature. The strains to be tested were then solidly streaked out, forming a cross on the surface of the medium, and a filter paper disc containing 10 µg penicillin G was placed on the centre of the cross. Following half an hour of prediffusion at room temperature, the plates were incubated at 36 C in a 5 per cent CO atmosphere, and read 18 hours later

The plate dil tion sensitivity tests were also performed on the chocolate" agar medium used in the disc method for sensitivity testing Four-fold dilutions of penicillin G were incorporated into the medium in concentrations from 512–0 03 µg/ml medium. The inoculum was prepared from 18 hours' culture on selecti chocolate agar suspended in physiological saline and 50-fold dilutions were made, giving 3 different inoculate concentrations The plates were inoculated on the surface and applied by a loop calibrated to deliver 0 001 ml, thus producing a round spot of approximately 6 mm diameter From the highest dilution, 0 001ml was also spread on the surface of the medium without penicillin with a iew to calculating the number of living gonococci in the dil tions. The incubation and reading of the plates was done as above mentioned.

Results

The results of the testing for penicillinase production are seen in Fig. 1 and Fig. 2 which show that strain Gc 16634 is powerful penicillinase producer

We also tested 10 recently isolated gonococcal strains which showed "reduced sensitivity" to penicillin G by the paper disc sensitivity test, but w were not able to detect penicillinase production.

Using the plate dilution sensitivity test,—Table 1 —the most concentrated inoculum showed uninhibited growth on the medium containing 32 µg penicillin G/ml whereas the corresponding penicillin G concentration in the medium used for the most diluted inoculum was only 0 5 µg/ml

This considerable ariation in sensitiv ty parallel with the bacterial population density in the test, indicates that the resistance of our gonococcal strain to penicillin probably is based ma ly on the penicillinase production

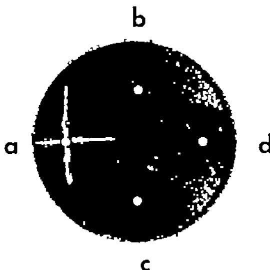


Fig 1 Test for penicillinase production. The medium (chocolate agar) was seeded with a penicillin sensiti strain of *St ph aureus* (P209) Strains to be tested were streaked crosswise and discs, each containing 10 µg penicillin G were placed in the centre of the cross t a, b and c. At a, control using a penicillinase producing *Staph. aureus* shows the typical picture of penicillinase production. At b, the strain Gc 16634 shows that the penicillin in the disc has been rendered ineffective allowing the sensiti staphylococcus to grow without inhibition At c, a control using a common penicillin sensiti strain of *N gonorrhoeae* gives no reduction of the growth inhibition zone, but there is a limitation in the zone at about 1 o clock caused by the Gc 16634 streaked out at d. At d, a disc containing 30 µg cephalotin was placed on the Gc 16634 cross. It is seen that the effect of the cephalothin on the seeded *Staph. au eus* is reduced to about the same degree as the effect of penicillin G is reduced by the penicillinase producing *Staph. au eus* at a.

Discussion

With the exception of the described penicillin resistant strain Gc 16634 w ha nc been able to detect penicillinase productio gonococcal strains which showed reduced sensitiv ty penicillin G by the paper disc test. N her could *Sparling et al.* (5) find evidence of penicillinase

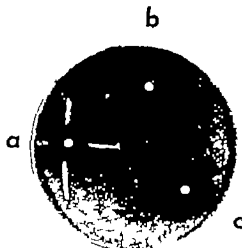


Fig 2 As in Fig. 1 but at a, b and c, discs containing 10 μg methicillin were used. At a the cross was made by a penicillinase producing *Staph. aureus* and no distinct deformation of the inhibition zone is seen, confirming that methicillin is highly resistant to staphylococcal penicillinase. At b the cross was made by Gc 16634 and the methicillin is rendered ineffective to such a degree that no inhibition zone is seen. At c, the cross was made by an ordinary penicillin sensitive gonococcal strain, and no reduction of the inhibition zones is seen.

production in *N gonorrhoeae* of low level resistance to penicillin and, as far as we know penicillinase producing strains of *N gonorrhoeae* have not been registered before.

The emergence of penicillinase producing *N gonorrhoeae* is proportionate to the discovery a couple of years ago of some strains of *Haemophilus influenzae* which were resistant to ampicillin, the resistance being found to be caused by production of penicillinase (2) Throughout the years, *H influenzae* strains had always been found to be sensitive to ampicillin.

As the penicillins, especially penicillin G and ampicillin, are considered the drugs of choice in the treatment of gonorrhoea, the appearance of a penicillinase producing gonococcal strain is disquieting.

The characteristics and the origin of the penicillinase remain to be elucidated.

TABLE 1 *Testing of the Sensitivity of Gonococcal Strain Gc 16634 to Penicillin G Using the Plate Dilution Method Inoculations with Different Bacterial Density Inoculation Volume 0.001 ml*

Concentration of penicillin G in the medium, μg/ml	No. of living gonococci/ml inoculate		
	1.8×10⁷	6×10⁴	2×10⁴
512	–	–	–
128	–	–	–
32	+++	–	–
8	+++	–	–
2	+++	++	–
0.5	+++	+++	++
0.125	+++	+++	+++
0.03	+++	+++	+++
0.00	+++	+++	+++

+++ = uninhibited growth, ++ = slightly weaker growth, – = no growth.

Addendum Since this paper was written it has been reported from the Center for Disease Control, Atlanta, Georgia (United States of America) that for the first time gonococci producing penicillinase have been isolated from several patients who had contracted gonorrhoea outside the United States. This information was given in the Weekly Epidemiological Report of 17 September 1976, issued by the World Health Organization, Geneva. In "The Lancet of 25th September 1976, p. 656, *I Phillips* describes the isolation of a penicillin resistant penicillinase-producing gonococcus in London, and p. 657 *H A Ashford R G. Golash & I G Hemming* report the isolation of a similar gonococcal strain in California.

We are indebted to *L. O F ekholm* of the Methodology Department, for the photographs.

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YERSINIA PSEUDOTUBERCULOSIS AS THE CAUSE OF SEPTICAEMIA IN A PATIENT WITH AMYLOIDOSIS

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Bevanger L. *Yersinia pseudotuberculosis* as the cause of septicaemia in a patient with amyloidosis. Acta path. microbiol. scand. Sect. B, 84 461–462, 1976

Septicaemia caused by *Y pseudotuberculosis* in a female patient, aged 61 is reported. The patient suffered from amyloidosis with extensive infiltration of liver spleen, and kidneys. While under treatment with corticosteroids and azathioprin, *Y pseudotuberculosis* serotype IA, was isolated from each of 6 blood cultures. The infection responded favourably to treatment with ampicillin. The development of *Y pseudotuberculosis* septicaemia owing to impairment of the defence mechanisms by the underlying disease and the treatment given is discussed.

Key words *Yersinia pseudotuberculosis* septicaemia amyloidosis

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In man, *Y pseudotuberculosis* may be the cause of mesenteric lymphadenitis or septicaemia. The first condition is relatively common and benign, the latter condition is rare and has a high mortality rate. This report describes septicaemia due to *Y pseudotuberculosis* in a patient with amyloidosis.

Case Report

On November 19 1975 a 61-year-old woman was admitted to the Medical Department, Regional Hospital N-7000 Trondheim For several weeks she had been suffering from oedema of the legs, nausea and loss of weight. Examination revealed a pleural exudate ascites, enlargement of the liver massive proteinuria, and impairment of the renal functions Renal and liver biopsies demonstrated amyloidosis She was treated with corticosteroids and azathioprin In spite of this, her renal functions gradually deteriorated and peritoneal dialysis was started Fi week after admission she developed hyperpyrexia and six blood cultures were taken during three-day period *Y pseudot b e losis*, highly sensitiv to ampicillin, was isolated from all of he cultures (see below) The temperature normalized on treatment of the patient with this antibiotic. Subsequent blood cultures yielded no growth of *Y pseudotuberculosis* but *C albi ans* was isolated from two cultures taken four days before she died, on Jan. 19 1976 *Y pseudot berculosis* was not isolated from faeces. Necropsy demonstrated amyloidosis in most organs, including the liver spleen and the kidneys In addition, lesions due to *C albicans* were detected. Abscesses, characteristic of *Y pseudot berculos* infection, were not demonstrated

Bacteriology

The microorganism isolated from th blood cultures was a Gram negati e rod e ne growth on routine media after 18 hours ncubation N t ent broth cultures were uniformly turbid hen in cubated at 22 C, and motile d occ ingly or in diplo forms were present At 3 the cultures showed floccular growth I pe Im mobile rods in aggregates or hor ha wer found

Tests for beta-galactosidase (NPC lase and urease activ ty were posit A id I not gas, was produced from glucose maltose nd annitol The organism wa oxidase neg H S and indol were not produced. No growth appeared on Sim-

monads citrate medium. Tests for lysine and ornithine decarboxylase activity were negative. Determination of serotype showed that the microorganism belonged to *Y pseudotuberculosis* serotype IA. A guinea pig inoculated i p with a broth culture of the bacterium, died within 2 days. Necropsy showed peritonitis and nodules in the liver and spleen. *Y pseudotuberculosis* was cultured from these sites. Gram-stained smears from the peritoneal exudate showed numerous granulocytes and macrophages, the latter being packed with bacteria.

Serum from the patient, taken at the time when blood-cultures were positive, agglutinated the patients own microorganism to a titre of 3?0 The antigen used was bacteria grown at ?2 C in a nutrient broth, and then treated with formaldehyde.

Comments

Y pseudotuberculosis is a common animal pathogen, sometimes causing epizootics. It has been isolated from guinea pigs, mice rats, rabbits, cats and other animals, and from birds (4) In man, infection usually presents itself as an acute mesenteric lymphadenitis. Generalized infection with septicaemia is rare Less than 50 cases have been reported (5 6) In both forms of the disease, the portal of entry is assumed to be the bowel Most observations indicate predisposition to septicaemia in patients with chronic liver diseases (cirrhosis, alcoholism, haemochromatosis) Such conditions may enable the microorganism to bypass the reticuloendothelial system of the liver thereby allowing dissemination via the peripheral blood (2, 3 5 6) Disordered iron metabolism has also been proposed as a predisposing factor (1)

Our patient suffered from amyloidosis with extensive infiltration of the liver spleen and kidneys. In addition she received treatment with immunosuppressive drugs. The underlying disease and the treatment given have probably resulted in severe disturbance of her defence mechanisms against infectious agents. This may explain why she acquired septicaemia by *Y pseudotuberculosis* and finally by *C. albicans*.

The author wishes to thank Dr *J Lassen* National Institute of Public Health, Oslo, Norway for serotyping the bacterial strain isolated, and Dr *J Hed*, Medical Department, Regional Hospital, Trondheim, Norway under whom the patient was admitted.

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